SYNTHESIS OF NUCLEOSIDE ANALOGUES AND FLUORESCENT LABELS FOR DNA SEQUENCING AND OTHER APPLICATIONS IN BIOTECHNOLOGY

A Thesis

by

MIHAELA NARCISA PREDESCU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Chemistry

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Approved by:

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ABSTRACT

Synthesis of Nucleoside Analogues and Fluorescent Labels
for DNA Sequencing and Other Applications
in Biotechnology. (August 2005)
Mihaela Narcisa Predescu, B. S., University of Bucharest
Chair of Advisory Committee: Dr. Emile Schweikert

Two pyrrolo[2,3-d]pyrimidines and a nucleoside analogue have been prepared. The nucleobases were obtained by spontaneous cyclisation of 4-aminopyrimidyl-acetaldehydes as an acetal. The adenosine analogue has been made by glycosylation of a suitable sugar derivative with the corresponding heterocyclic base. This nucleoside can be converted into fluorescently-labeled chain-terminating substrates for DNA polymerase after subsequent triphosphorylation and coupling to through-bond energy transfer systems.

Fluorescein-based donor components to be incorporated into through-bond energy transfer systems have been prepared. The synthesis of 5-ethynylfluorescein-(5-*tert*-butoxycarbonyl)-pentyl ester has been executed in five steps from 1,3-dihydroxybenzene and phthalic anhydride. The donor fluorescein carboxylate has been alkylated with the *tert*-butyl ester of 6-bromohexanoic acid to provide a handle for attachment to biomolecules. In the context of regioisomerically pure halofluoresceins, besides the synthesis of pure regioisomers of bromofluorescein derivatives, 5-iodosulfofluorescein and pure regioisomers of 5-nitrofluorescein diacetate, as an intermediate in the synthesis of 5-iodofluoresceins, have been synthesized.

New rhodamine-based acceptor components with extended aromatic systems have been prepared from an affordable starting material, tetralin. The attempts to isolate them via repeated recrystallizations and flash chromatography have been unsuccessful. However, these pyrylium cations are expected to fluoresce at longer wavelengths.

ACKNOWLEDGEMENTS

I would like to thank Dr. Emile Schweikert for his guidance, Drs. Marian Hyman, Kenn Harding, and Nancy Amato for serving on my advisory committee. I would also like to thank my family, my parents for their support and for never discouraging me, my sister, and brother-in-law for their support and helpful discussions, and my friends.

I would especially like to thank God for giving me the strength and endurance to complete this work.

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LIST OF SCHEMES

LIST OF ABBREVIATIONS

AcOH	acetic acid
BODIPY	boron dipyrrylmethane
$CHCl_3$	chloroform
CH_2Cl_2	dichloromethane
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
ddT	dideoxythymidine
DMF	*N*,*N*-dimethylformamide
DNA	deoxyribonucleic acid
EtOAc	ethyl acetate
EtOH	ethanol
ET	energy transfer
FRET	fluorescence resonance energy transfer
HCl	hydrochloric acid
MeCN	acetonitrile
MeOH	methanol
NBD	7-nitrobenzo-2-oxa-1-diazole
Py	pyridine
Taq	thermus aquaticus

CHAPTER I

INTRODUCTION

1.1 DNA Sequencing

DNA sequencing is one of the fundamental techniques of modern molecular biology and it enables us to identify the sequence of nucleotides of the human genome. Two methods to sequence DNA were developed: the Maxam-Gilbert chemical degradation method, that describes a process whereby terminally labeled DNA molecules are chemically cleaved at single base repetitions,[1] and the chain-terminator method of Sanger that involves the chain termination of DNA synthesis by the incorporation of 2′,3′-dideoxynucleoside triphosphates which act as chain-terminating inhibitors of the DNA polymerase.[2]

Sanger dideoxy DNA sequencing is the most commonly used method for determining the nucleotide sequence of a DNA fragment. It is based on the use of dideoxynucleotides in addition to the normal nucleotides found in DNA and it takes advantage of the chain terminating ability of dideoxynucleoside triphosphates and the ability of DNA polymerases to incorporate them almost as well as the natural substrate of DNA polymerase, deoxynucleoside triphosphates.

In the chain-terminator technique (Figure 1.1), the oligonucleotide being sequenced is replicated by the enzyme using a suitable primer and the four 2′-deoxynucleoside triphosphates. In addition, the 2′,3′–dideoxynucleoside triphosphate of one of the bases is added to the reaction mixture and the reactions are performed in four different tubes. As the DNA is synthesized, nucleotides are added in the growing chain by the DNA polymerase. However, when a dideoxynucleotide is incorporated into the chain in place of a normal nucleotide, the chain is terminated by only one of the four

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This thesis follows the style and format of *The Journal of Organic Chemistry*.

dideoxynucleoside triphosphates as dictated by the template DNA because of the absence of the hydroxyl group on the 3′-carbon. A series of truncated fragments are produced. They have different lengths due to the random incorporation of the dideoxynucleotides. The fragments produced by all four dideoxynucleotides (in separate reactions) are then separated by electrophoresis according to their size in four separate lanes of a slab gel. Each band differs in size and mobility by one nucleotide and the terminating nucleotide is identified by knowing which lane contains the band. In this way, visualization of the bands permits deduction of the sequence of the DNA template.

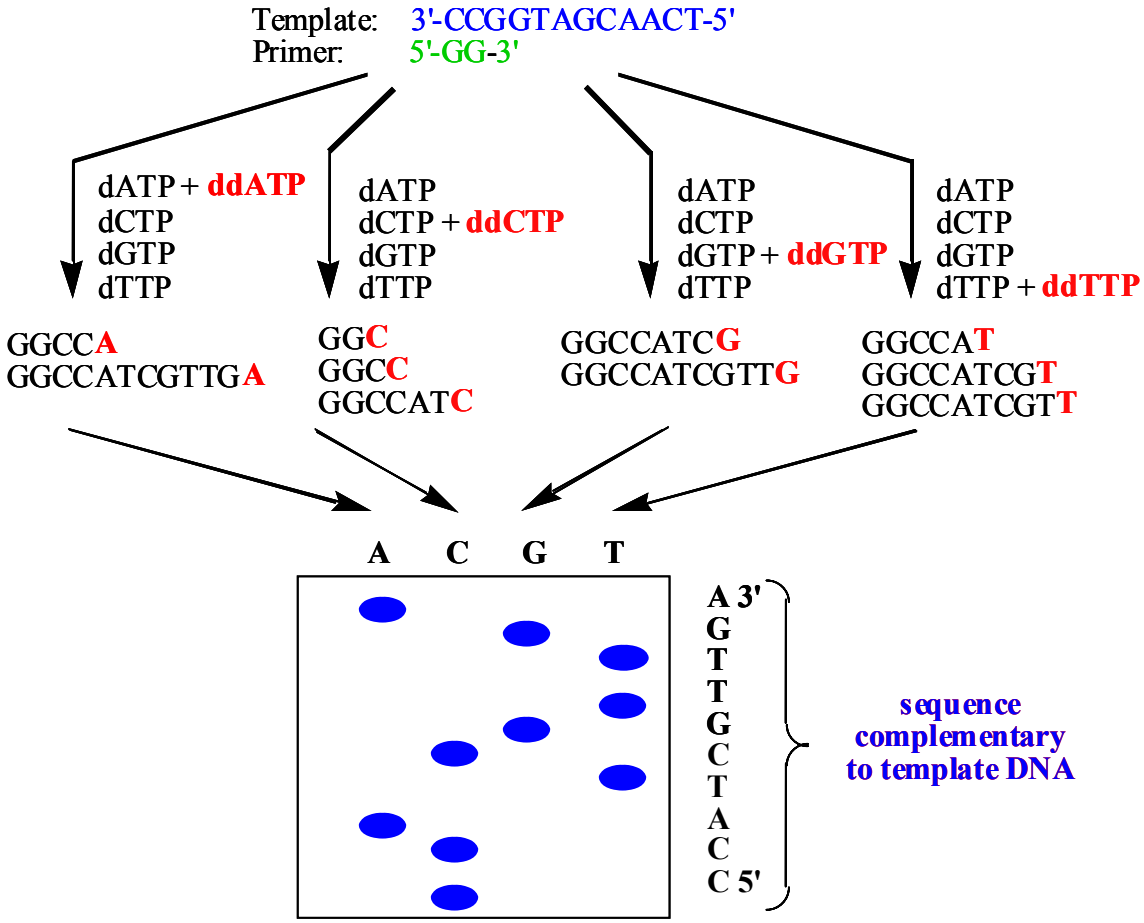


Figure 1.1. A flow diagram of the chain-terminator method of DNA sequencing.[3]

The fragments are visualized using radioisotope or fluorescence labeled reagents. Radioisotopic labeling was originally used to visualize the oligonucleotide fragments. However, fluorescence detection has replaced radioisotopic detection[4] because of the advantages that fluorescence-detection has. Fluorescence labeling permits real-time detection and is more amenable to automated data collection and analysis. In addition, the radioisotopes are unstable, expensive, and their handling and storing may be hazardous and difficult.

With the latest advancement in technology, automated sequencing has been developed, and it is based on the same principles of Sanger's method. With the automated procedures more DNA can be sequenced in a shorter period of time and the reactions are performed in a single tube containing all four dideoxynucleotides, each labeled with a different color fluorescent dye.

As in Sanger's method, the DNA is separated by capillary electrophoresis, but they are all run on the same lane, not on four different ones. Since the four dyes fluoresce at different wavelengths when irradiated with an appropriate laser source, the identity of each nucleotide can be determined according to the wavelengths at which the fluorescent dyes emit.

1.2 Chain Terminators in DNA Sequencing

There are two variations of automated fluorescence-detected DNA sequencing: dye-labeled primer sequencing[4-7] in which the fluorescent dyes are attached to the 5′-end of the primer oligonucleotide, and the dye-labeled terminator sequencing in which the fluorescent dyes are attached to the terminating dideoxynucleoside triphosphates.[8] The dye-labeled terminator sequencing has several advantages over dye-labeled primer technique. The major drawback of the dye-labeled primer sequencing is the requirement for four separate extension reactions. In addition, four fluorescently-labeled primers need to be prepared for each template and fragments resulting from misincorporation of deoxynucleotides are visible to the detector. The primary advantage of the dye-labeled terminator method is that only one extension reaction is required for each template.

Moreover, the DNA fragments that are terminated by a deoxynucleotide in place of a dideoxynucleoside (i.e. false terminations) are not fluorescent since these products are not fluorescently-labeled. The dye-labeled terminator sequencing has disadvantages as well, the primary one being the limitation of the polymerase enzymes that can be used since these modified dideoxynucleotides are not accepted by many polymerases. However, many attempts towards elaborating enzymes that will satisfactorily integrate dye-terminators and that will be less discriminating against dideoxynucleotides have been made.[9-11] The dye-labeled terminator technique uses 2′,3′-dideoxynucleoside triphosphates (Figure 1.2) which act as chain-terminating inhibitors of the DNA polymerase. They are modified nucleotides that can be incorporated on the 3′-end of a template DNA, but once incorporated, prevent the addition of further nucleotides. This occurs because a phosphodiester bond cannot form between the dideoxynucleotide and the next incoming nucleotide since the dideoxynucleotide lack the hydroxyl group on the 3′-carbon, and thus the DNA chain is terminated.

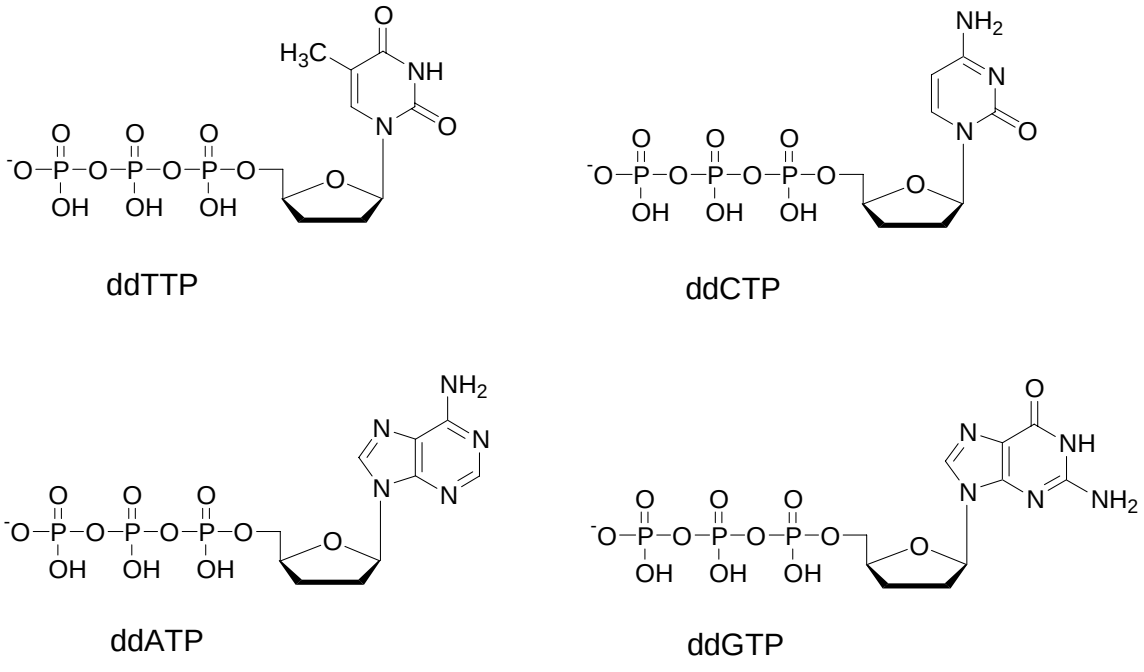


Figure 1.2. 2′,3′-Dideoxynucleoside triphosphates.

Prober *et al.* at DuPont developed the first set of dye-terminators for DNA sequencing. They are comprised of a succinylfluorescein coupled to a dideoxynucleoside triphosphate via an acetylenic linker. Their chemical structures along with their absorption and emission maxima are shown in Figure 1.3.

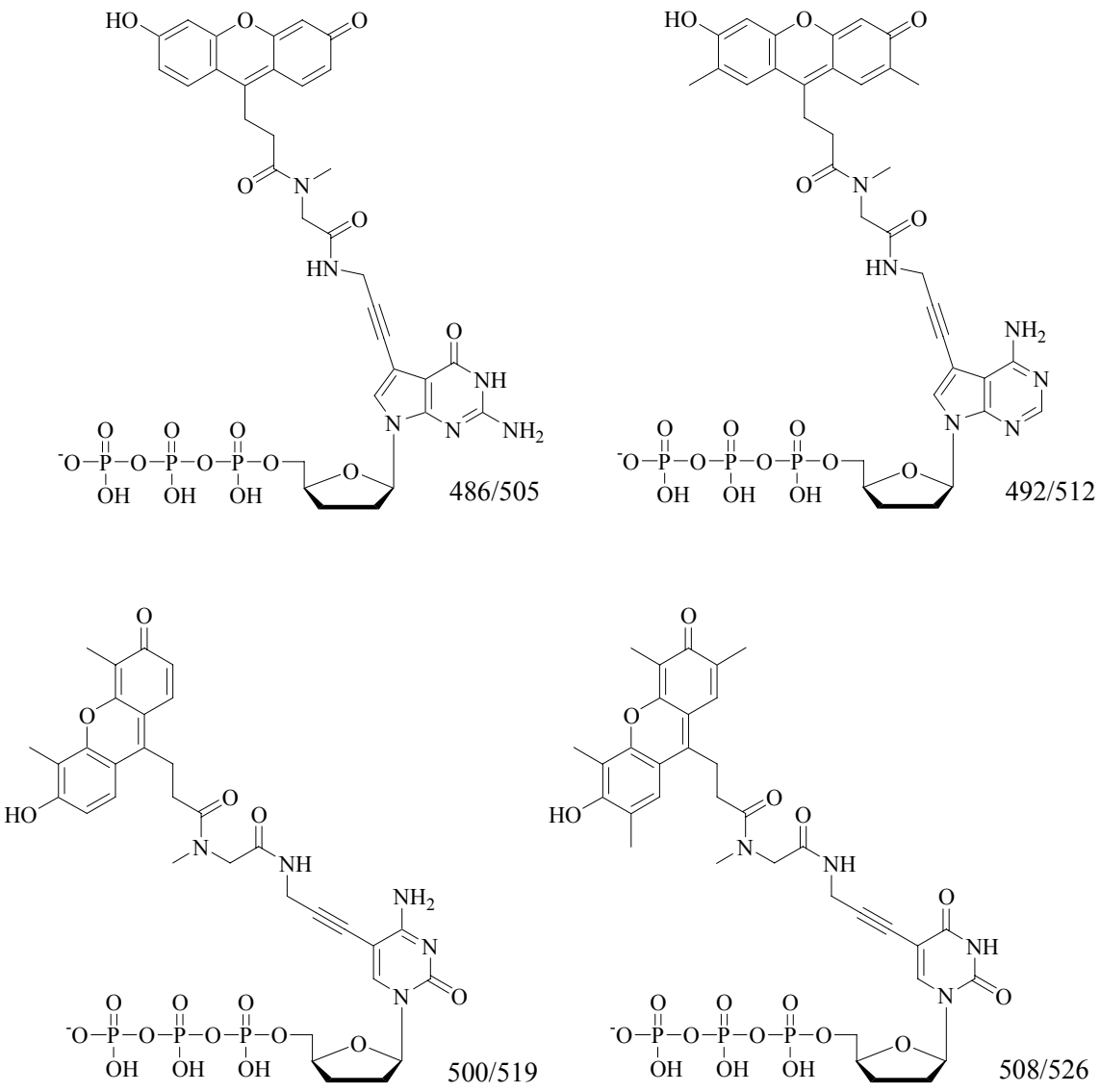


Figure 1.3. The first dye-terminators.[8]

These chain-terminators were prepared from succinic anhydride and a suitably substituted resorcinol and were incorporated by both Avian myeloblastosis virus reverse transcriptase and T7 DNA polymerase.[8] Since their discovery until today, the design of most of the dye-terminators is based on Prober's terminators - the fluorescent dyes are attached to the 5-position of pyrimidine and the 7-position of 7-deazapurine dideoxynucleosides via a propargylamide linker. The total synthesis of a nucleoside analogue, 7-iodo-2′,3′-dideoxy-7-deazaadenosine, is described in detail in Chapter II of this thesis. Labeling these terminators with fluorescent dyes is quite important with respect to enzymatic incorporation, which makes the dye terminators to be quite expensive.

1.3 Fluorescent Dyes in DNA Sequencing

The fluorescent dyes that are attached to the 2′,3′-dideoxynucleoside triphosphates are critical to the sensitivity of DNA sequencing. The major criteria which are used in the selection of these fluorophores are as follows: (1) their absorption and emission maxima have to be in the visible region of the spectrum; (2) the four dyes must emit at wavelengths that are different enough to allow them to be distinguished spectroscopically, when irradiated by an excitation source that emits at a single wavelength; (3) the presence of the fluorophores should not affect the electrophoretic mobility of the DNA fragments. The ideal dyes should be highly fluorescent and should emit with similar intensities and with great resolution. The aim of this thesis is not to describe all of the dye systems developed for DNA sequencing, but the most significant ones.

The initial set of fluorescent dyes used in the original dye-primers was developed by Lloyd Smith and coworkers, who initiated the use of fluorescence detection in DNA sequencing in 1986. The structures of these dyes, 5-carboxyfluorescein, 7-nitrobenzo-2-oxa-1-diazole (NBD), tetramethylrhodamine, and Texas Red and their emission maxima are shown in Figure 1.4.

The emission maxima of these four dyes, which range from 520 nm to 595 nm, are in the visible region of the spectrum and are dispersed over a 75 nm wavelength range, which allow them to be differentiated spectroscopically.

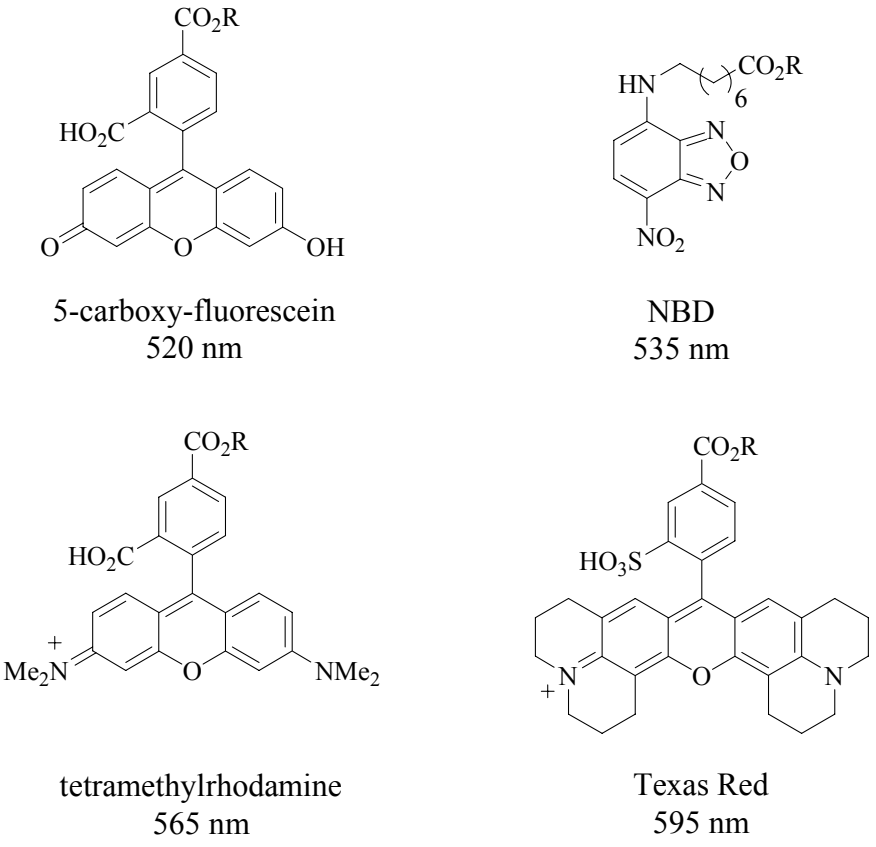


Figure 1.4. Dyes used in the original dye-primers.[4]

However, there are problems with original dyes. Their emission spectra overlap and not all dyes can be efficiently excited by a laser source that operates at a single wavelength. In addition, attachment of the dyes disturbed the electrophoretic mobilities of the labeled oligonucleotides.

Further research has been undertaken to find modified dyes with improved fluorescent characteristics. The structures of a common set of fluorescent dyes used to label the primers for DNA sequencing are shown in Figure 1.5. These fluorescent dye-

primers bear a fluorescein-based donor (FAM) attached to the 5′-end and other fluorescein and rhodamine derivatives connected to a modified thymidine residue in the primer sequence as acceptors. Since these primers use fluorescence energy transfer to improve the spectroscopic properties of the label, they are called energy-transfer primers.

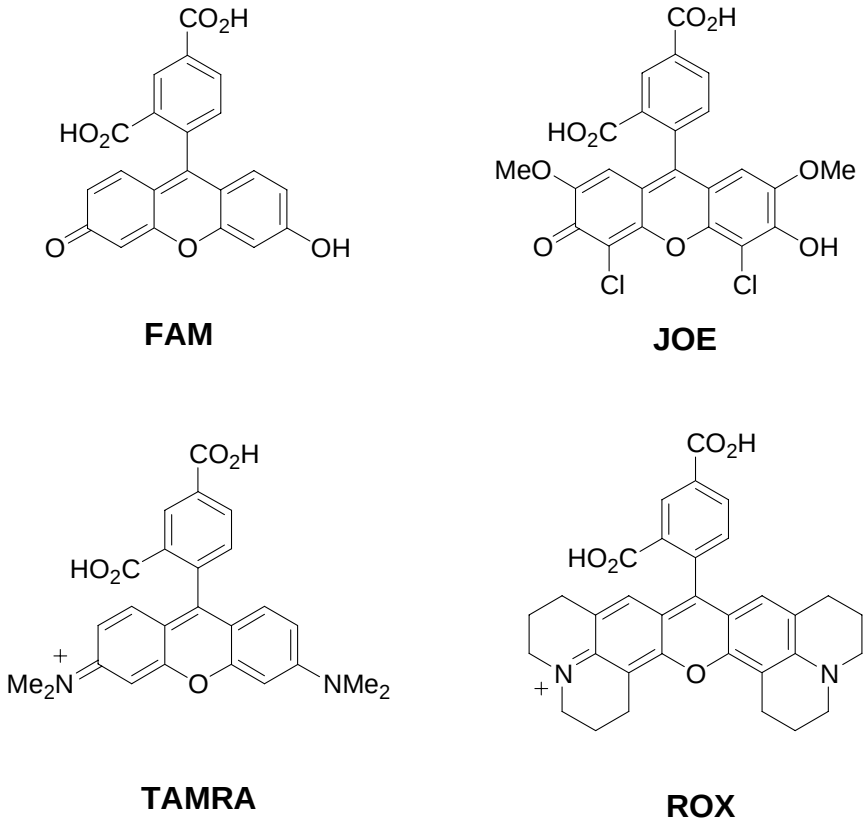


Figure 1.5. Dyes used in energy-transfer primers.[6]

The emission spectra of these modified dyes are reasonably different, which would allow good spectral differentiation. However, when the dyes are excited by a laser source that operates at a single wavelength of 488 nm (the excitation from an Argon-laser), they emit with unequal intensities because not all these four dyes absorb

efficiently when excited by the same excitation source. The fluorescence intensities depend on the amount of light absorbed at the excitation wavelength.

Metzker and Gibbs replaced the standard set of fluorescein and rhodamine dyes with boron dipyrrylmethane (BODIPY) dyes (Figure 1.6).

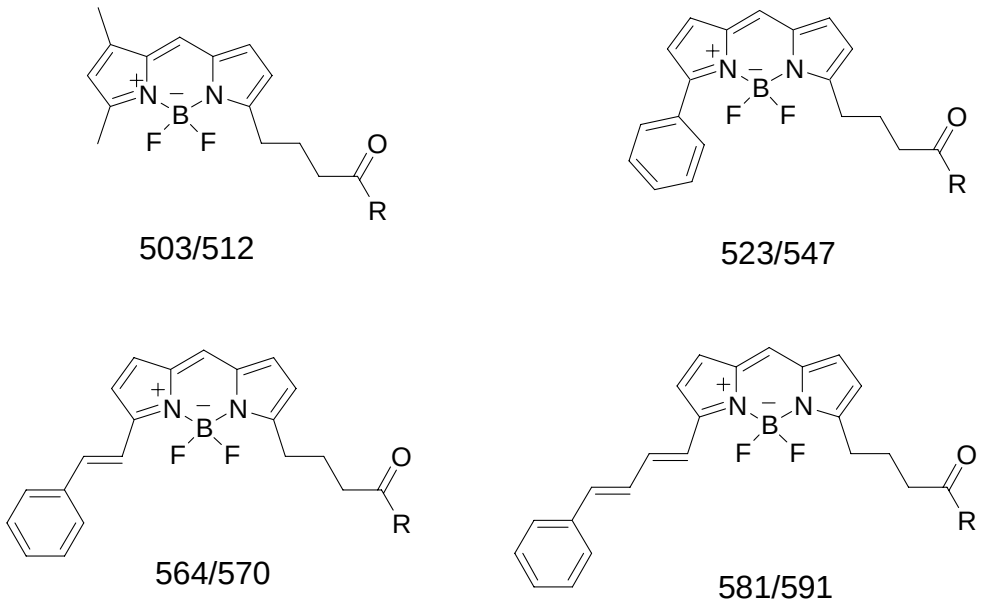


Figure 1.6. BODIPY dyes used in Metzker's energy transfer primers.[7]

These dyes are based on an atypical fluorophore which contains boron and were used to label DNA primers. BODIPY dyes have several advantages over conventional fluorescein and rhodamine dyes. Their emission spectra are narrower, thus increasing the accuracy of base calling. In addition, the BODIPY-labeled primers overcome electrophoretic mobility problems experienced with the previous dyes utilized in sequencing techniques.

Prober and coworkers have developed a DNA sequencing system where the dideoxynucleoside triphosphate terminators (Figure 1.3) have been labeled with four different succinylfluorescein dyes.[8] These single dye systems were spectrally resolved

and their attachment to the DNA fragments resulted in almost identical electrophoretic mobilities.

Although single dyes systems offer the above advantages, not all four dyes can be effectively excited when irradiated by a laser source that emits at a single wavelength. Consequently, it is difficult to observe several fluorescently-tagged components at the same time because the dyes that emit at longer wavelengths absorb at the excitation wavelength less efficiently, and thus have decreased fluorescence intensities compared to the short wavelength fluorescent dyes. The most commonly used DNA sequencers employ an argon laser and the dyes having longer wavelength emission do not have satisfactory absorbance at 488 or 514 nm. A partial solution to overcome these drawbacks is using fluorescence resonance energy transfer (FRET) between two dyes, one dye-component as the donor that absorbs energy from the excitation source and the other dye-acceptor as the acceptor that fluoresces at a longer wavelength. In the context of FRET systems, the excited-state energy from the initially excited donor is transferred, without emission of a photon, to an acceptor, resulting in emission of a longer wavelength photon from the acceptor. The rate of energy transfer depends mainly on the extent of overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, the distance between the fluorophores (which is inversely proportional to the sixth power of the distance, r, between the donor and the acceptor), and their relative orientations.

Mathies *et al.* have applied the energy transfer concept to four-color fluorescence detected sequencing to show that the energy transfer primers are better than single dye-labeled primers.[6] The donor component was attached to the 5′-terminus of the oligonucleotide primers. The acceptor components were connected to the primary amine group on the modified base. The donor dye chosen for Mathies' experiment was 5-carboxyfluorescein (FAM), and acceptor dyes selected were 2′,7′-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (TAMRA), and 6-carboxy-X-rhodamine (ROX) (Figure 1.5). These energy transfer

systems showed enhanced signal strengths compared to the single dye-labeled primers. However, they suffer from having disproportionate mobility shifts.

In 1988, Lee and coworkers at Applied Biosystems analyzed a dye dimer named Bifluor-1 (Figure 1.7), synthesized and provided by Molecular Probes, Inc.

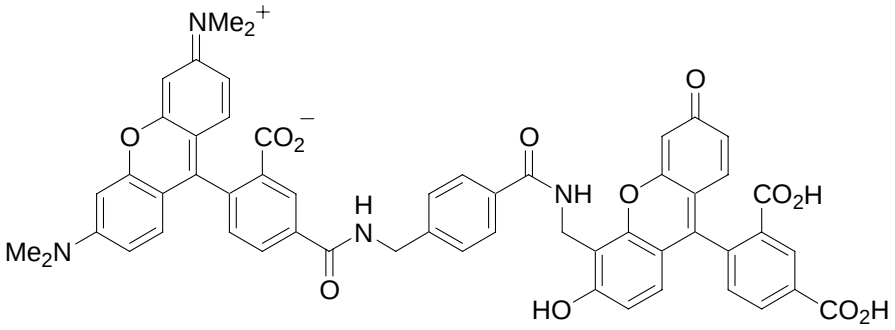


Figure 1.7. Bifluor-1.[12]

This compound is comprised of 4′-aminomethyl-5-carboxyfluorescein as a donor and 5-carboxytetramethylrhodamine as an acceptor connected via an aminomethylbenzoic acid linker. Their analysis showed that the absorption spectrum of Bifluor-1 was the cumulative spectra of the two dye fragments. Moreover, excitation of the dimer produced fluorescence characteristic of only the acceptor component, tetramethylrhodamine, meaning that the energy was efficiently transferred between the two dye components. However, since Bifluor-1 proved to be no brighter than the tetramethylrhodamine portion alone, Lee's effort was then directed towards improving its spectral properties by preparing diverse combinations of fluorescein and rhodamine components of the original Bifluor-1. Consequently, a set of four dichlororhodamine dyes was prepared by adding 4, 7-dichlorosubstituents to the rhodamine dyes. They have sharper emission profiles and emit at longer wavelengths than the unsubstituted rhodamines. These energy transfer systems have been initially designed for use in dye-primer sequencing. The same year, they extended the advantages of the energy transfer

to dye-terminators and designed the "Big Dyes" (Figure 1.8). These dye-terminators proved to be superior to previous dye-terminators with respect to signal-to-noise ratios and even peak patterns.

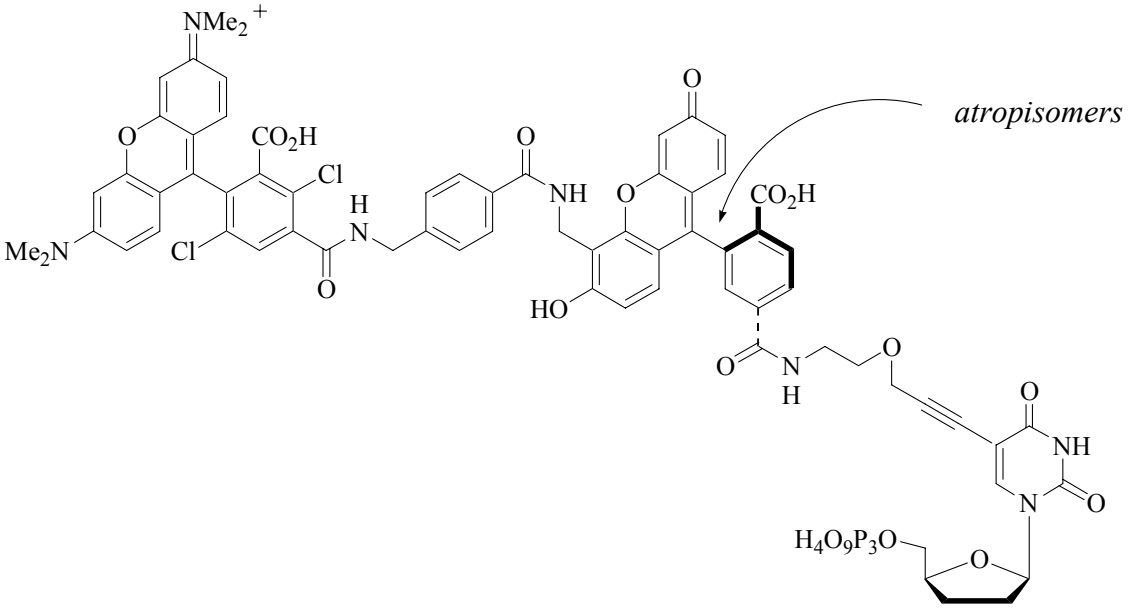


Figure 1.8. Structure of the ddT-Big Dye terminator.[5]

However, the Big Dyes have a drawback - they are all chiral and show atropisomerism. When they are attached to the dideoxynucleotides, a mixture of diastereoisomers having non-identical electrophoretic mobilities forms. To alleviate this problem, Lee *et al.*[13] prepared dyes optically pure by coupling the corresponding fluorescein derivative with (-) menthyl chloroformate to yield two diastereoisomers which were separated by HPLC.

Although the fluorescence resonance energy transfer systems proved to be a significant improvement over the single dye systems, unfortunately, the resolution of emissions with FRET-based systems is still limited by the requirement that the fluorescence of the donor must overlap with the absorption of the acceptor. To alleviate this problem, Lindsey and coworkers developed through-bond energy transfer dyes wherein two fluorescent dyes, a donor and an acceptor, are connected via a conjugated

system.[14,15] The through-bond pathway does not require overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor as opposed to the Förster mechanism. Moreover, the rate of energy transfer involving conjugated linkers is not as constrained by the distance between the two fluorophores. Optimal through-bond energy transfer systems should contain a donor component that absorbs strongly when excited by a convenient excitation source, and an acceptor component that fluoresces strongly once the energy is transferred efficiently from the donor fragment via the conjugated linker. Selection of the linker is also critical. The linker does not have to allow the donor and acceptor components to become planar, otherwise each dye fragment would not retain its individual electronic properties and the system would behave as a single conjugated dye. However, a large deviation from planarity must be avoided in order for energy transfer to occur. Through-bond energy transfer systems have the ability to enhance both the resolution and fluorescence intensities when excited by a laser source that emits at a single wavelength.

A representative example of through-bond energy transfer systems that Lindsey developed is the one that employs a BODIPY dye as a donor component and a free base porphyrin as an acceptor, conjugated via metalloporphyrin linker (Figure 1.9). The efficiency of energy transfer of 76 % measured when exciting the BODIPY donor is superior to that expected from fluorescence resonance energy transfer (6 %).

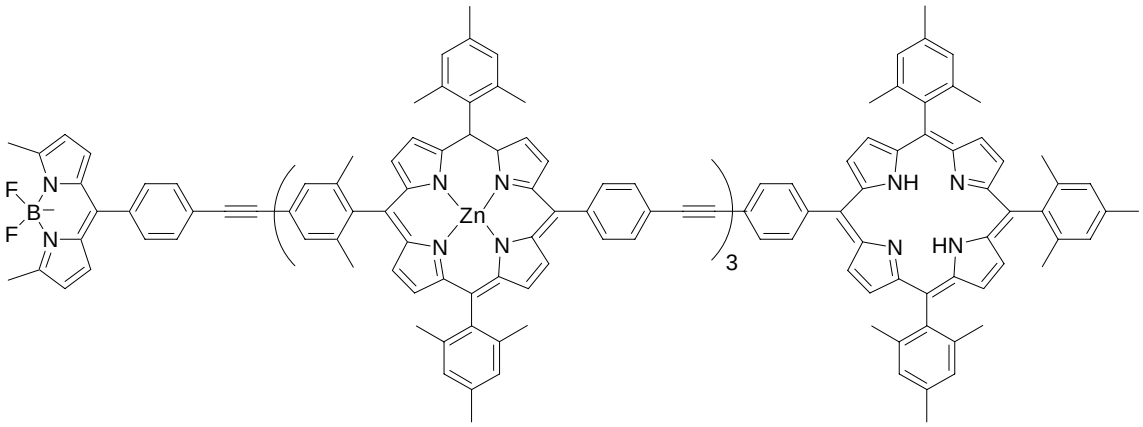


Figure 1.9. Lindsey's "molecular wire".[14]

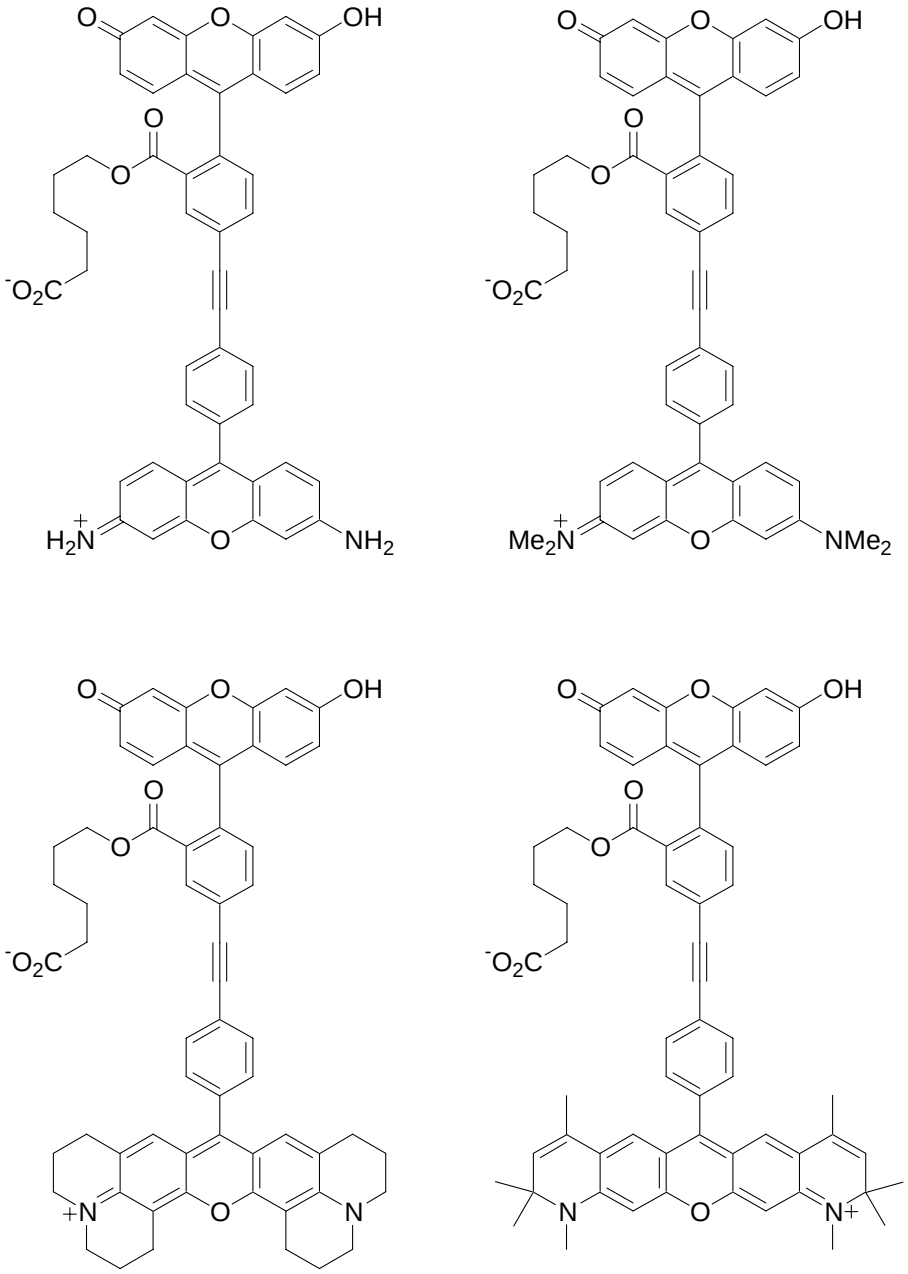


Figure 1.10. Through-bond energy transfer cassettes.[16]

A set of four through-bond conjugated cassettes useful for multiplexing has been developed by Jiao *et al*. All four systems employ fluorescein as a donor conjugated to rhodamines or extended rhodamines (Figure 1.10). The fluorescence spectrum of these

cassettes shows that their emission maxima are spread over a 78 nm wavelength range, which reflects a good resolution. As for the sensitivity, they fluoresce more brightly than the corresponding acceptor components when irradiated at 488 nm. The experiments to measure the rates of energy transfer are underway. However, these through-bond energy transfer systems might substitute for many fluorescent dyes used currently for DNA sequencing.

1.4 Summary

DNA sequencing is one of the fundamental techniques of modern molecular biology and it enables us to identify the sequence of nucleotides of the human genome. Of the two methods used for DNA sequence analysis, Sanger dideoxy DNA sequencing is the most commonly used. It is based on the use of dideoxynucleotides in addition to the normal nucleotides found in DNA and it takes advantage of the chain terminating ability of dideoxynucleosides triphosphates and the ability of DNA polymerase to incorporate them almost as well as the natural substrate of DNA polymerase, deoxynucleoside triphosphates. Fluorescence detection has replaced radioisotopic detection because it permits real-time detection and is more susceptible to automated data collection and analysis. In addition, the radioisotopes are unstable, expensive, and their handling and storing may be hazardous and difficult.

The fluorescent dyes that are attached to the 2′,3′-dideoxynucleoside triphosphates are critical to the sensitivity of DNA sequencing. The ideal fluorophores should comply with the following requirements: (1) their absorption and emission maxima have to be in the visible region of the spectrum; (2) the four dyes must emit at wavelengths that are different enough to allow them to be distinguished spectroscopically when irradiated by an excitation source that emits at a single wavelength; (3) the presence of the fluorophores should not affect the electrophoretic mobility of the DNA fragments. The ideal dyes should be highly fluorescent and should emit with similar intensities and with great resolution. Developments in fluorescent dyes showed that the through-bond energy transfer systems are superior to the single dyes and

FRET-based systems since they have the ability to enhance both the resolution and fluorescence intensities when excited by a laser source that emits at a single wavelength. Through-bond energy transfer systems have applications not only in DNA sequencing, but also other applications in biotechnology, if optimized to provide longer wavelength. One of the challenging applications is intracellular imaging which focuses on defining the role of the numerous proteins in the cellular processes associated with health and disease and can be facilitated by developments in fluorescent dyes and proteins.

CHAPTER II

NUCLEOSIDE ANALOGUES

2.1 Introduction

Nucleosides and their analogues are of great importance in molecular biology. They can be used as therapeutic agents possessing antiviral and anticancer activity, in detection of nucleotide mutations, and in DNA sequencing.

The most popular terminators of enzymatic DNA synthesis are 2′,3′-dideoxynucleosides that are the core for currently used sequencing procedures. Since the synthesis of the first pyrimidine 2′,3′-dideoxynucleoside, 3′-deoxythymidine, by Michelson and Todd[17] in 1955, various 2′,3′-dideoxynucleoside analogues have been synthesized. In 1964, Robins and Robins[18] described the synthesis of the first purine dideoxynucleoside, 2′,3′-dideoxyadenosine, and anticipated that their 5′-triphosphates might act as potential chain-terminating inhibitors of the DNA polymerases dideoxynucleotide because they lack the hydroxyl group on the 3′-carbon. That hypothesis was developed later on by Sanger into the well known dideoxy method of DNA sequencing.[2]

The continuing search for optimal DNA sequencing reagents is due to the variety of polymerases, nucleotide analogues, and reaction conditions available. In general, the enzymes used for sequencing DNA are highly substrate selective. DNA polymerases can be classified in three families: A, B, and C. The most widely used enzymes in DNA sequencing are DNA polymerases of Family A (e.g. E. coli pol I, T7, *Taq* DNA polymerases). One of the first set of dyes developed for T7 DNA polymerase contained a propargylamino linker connected to fluorescein dyes. However, the dye terminators developed for *Taq* Pol I contained rhodamines, since fluorescein terminators were poorly incorporated.[9] DNA polymerases of other families have also been examined for their ability to incorporate modified nucleotides. Family B Archaeon incorporate acyclic nucleoside triphosphates preferentially over dideoxy terminators, while the Family A

DNA polymerases *Taq* have a preference for dideoxy terminators. An explanation for this preference is that there are considerable differences in sugar recognition in these two families of polymerases.[11] AcycloTerminators consist of an acyclic nucleoside triphosphate looking like the herpes drug Acyclovir, an acyclic analogue of guanosine, 9-[(2-hydroxyethoxy)methyl]guanine (Figure 2.1) attached to near-IR cyanine dyes. AcycloTerminators have the advantage that their synthesis is less laborious and affordable compared to the sugar-based dideoxynucleotides.

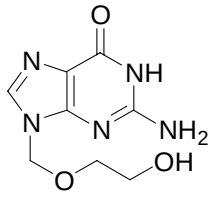


Figure 2.1. Acyclovir

The significant differences in enzymatic incorporation of the terminators noted in the examples mentioned above lead to the conclusion that it is difficult to predict structural or chemical basis by which terminators enhance enzymatic incorporation. However, there are two fundamental structure characteristics that the nucleoside analogues must maintain. When modifying the base, it is important to consider its ability to form hydrogen bonds with its complementary DNA base. On the other hand, when modifying the sugar component, the only requirement is the 5′-hydroxyl group which is engaged in phosphorylation. Consequently, there is more flexibility to vary the sugar moiety.

Attempts to find optimal nucleoside analogues with respect to both less laborious synthesis and ability to be enzymatically incorporated have been made. Thus Martinez *et al.* developed some acyclic nucleoside analogues and evaluated them as substrates for DNA polymerases (Figure 2.2). Although their synthesis is not laborious, the corresponding triphosphates were incorporated by the enzymes employed less efficiently than dideoxythymidine and dideoxyadenosine triphosphates.

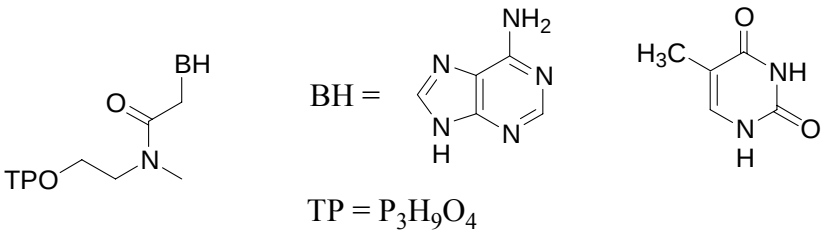
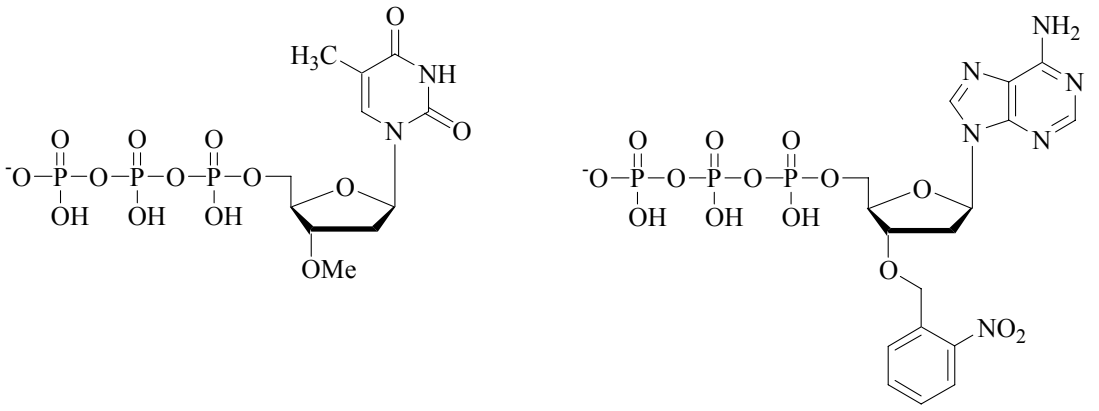


Figure 2.2. Acyclic nucleoside analogues.[19]

Other nucleoside analogues containing a blocking group at the 3′-hydroxyl group have been synthesized and evaluated for other approaches to DNA sequence analysis. These 2′-deoxynucleoside triphosphates looked like the dideoxynucleoside triphosphates with regard to the ability to terminate the DNA chain. Metzker et al. synthesized several 3′-*o*-nitrobenzyl nucleoside triphosphates (Figure 2.3) and tested them as substrates for several DNA replication enzymes. The results showed that the enzymes employed in the experiments efficiently incorporated the 3′-analogues. However, incorporation did not occur for any of the enzymes tested when a large fluorescent label was attached to the same position.



2′-deoxy-3′-*O*-methylthymidine 2′-deoxy-3′-*O*-(2-nitrobenzyl)adenosine

Figure 2.3. Metzker's 3′-blocked triphosphates.[20]

A few years later, other nucleoside triphosphates with both fluorescent and photolabile 3′-*O*-blocking groups were prepared and tested for incorporation.[21] The analogues were not incorporated by the enzymes employed, being too large to fit into the active site of the enzymes.

Although efforts have been made towards the synthesis of alternative nucleoside analogues to be converted into chain-terminators of the DNA polymerase, 2′,3′-dideoxyribonucleoside-5′-triphosphates are the most popular. Several positions are theoretically available for attachment of the fluorophores and for this reason, the position for modification of purines was uncertain. However, the position of substitution of the four bases is critical since it has an intense effect on polymerase activity. The instability and toxicity problems associated with the common 2′,3′-dideoxynucleosides were overcome by replacing the ordinary purine moiety by a 7-deazapurine base. While the 8-position of the purines is easily brominated, 8-substituted purine nucleosides show a preference for the *syn*-conformation at the N-glycosylic bond that does not make them appropriate for DNA polymerases.[9, 22] Moreover, alkylation at the 7-nitrogen would produce an unstable nucleoside.

2′,3′-Dideoxynucleosides are typically synthesized by multistep transformations from intact nucleosides,[23-26] involving deoxygenation reactions to 2′,3′-unsaturated dideoxynucleosides, which are then hydrogenated, or from 2′-deoxynucleosides via Barton-type deoxygenation reactions.[26-28] 7-Iodo-2′,3′-dideoxy-7-deazaadenosine can be synthesized by deoxygenation of the naturally occurring tubercidin, followed by regioselective mercuration/iodination as outlined in Figure 2.4.

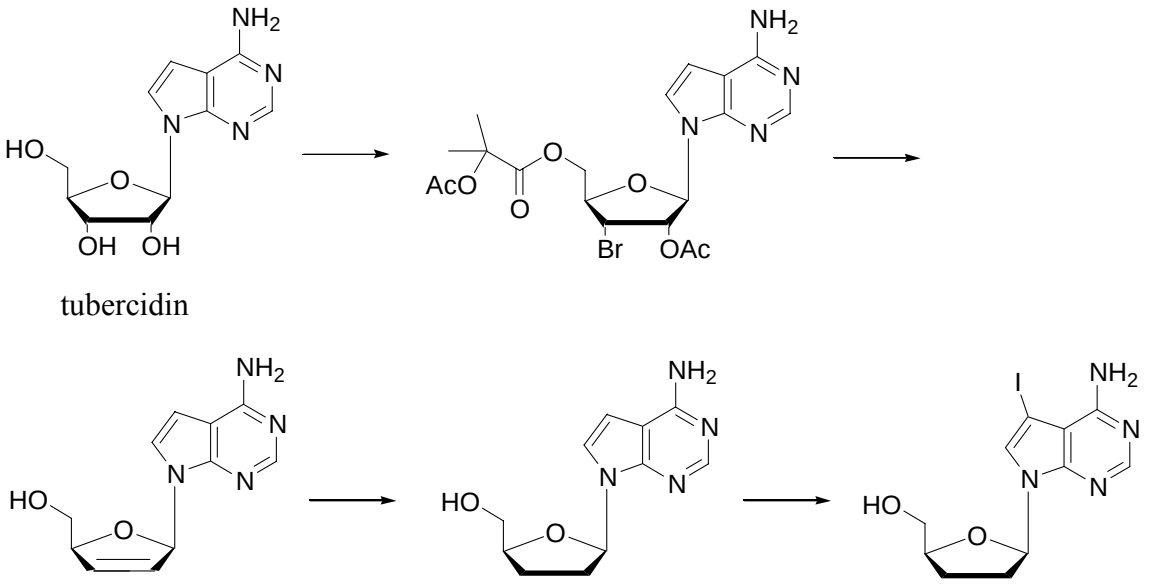


Figure 2.4. Synthesis of 7-iodo-2′,3′-dideoxy-7-deazaadenosine from tubercidin.[24,25]

However, there are alternative pathways to 7-iodo-2′,3′-dideoxy-7-deazaadenosine that do not employ tubercidin, an expensive fermentation product, but involve deoxygenation reactions of 2′-deoxynucleosides. This route is described in great detail in this chapter.

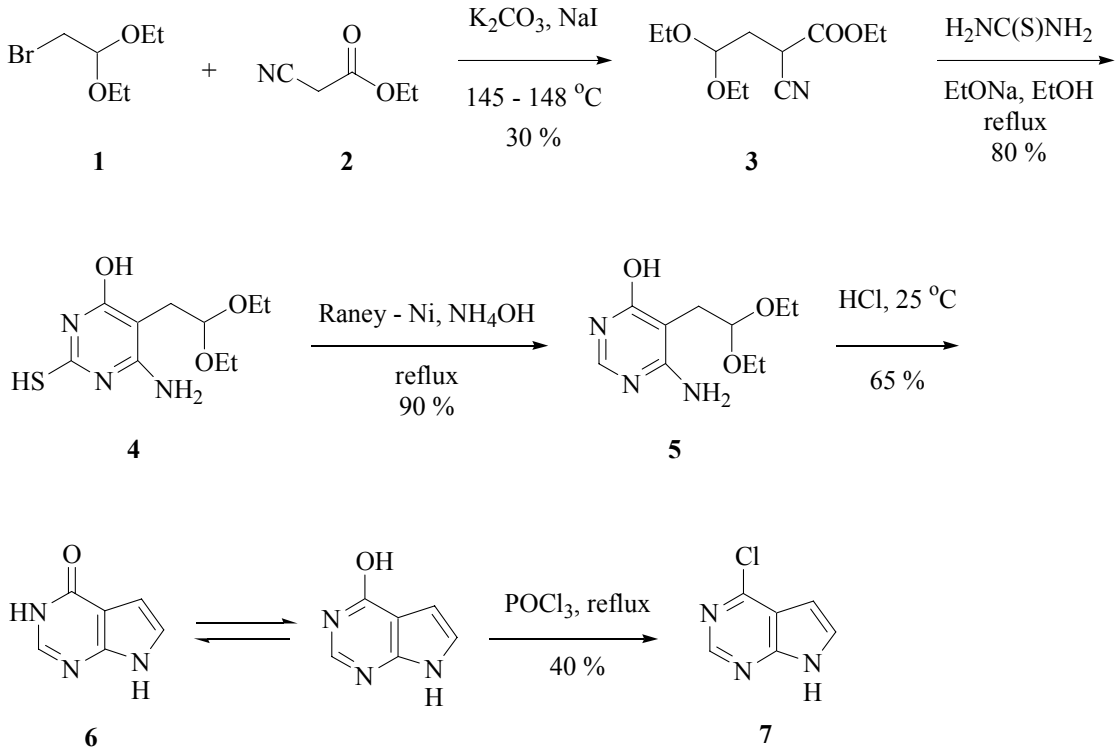
2.2 Total synthesis of 7-iodo-2′,3′-dideoxy-7-deazaadenosine

In a total synthesis of 7-iodo-2′,3′-dideoxy-7-deazaadenosine that was executed in 13 steps following Cocuzza's procedure,[28] 4-chloro-7*H*-pyrrolo[2,3-d] pyrimidine and 2-deoxy-3,5-ditoluoyl pentafuranosyl chloride were used as starting materials. However, owing to their unaffordable cost, they were synthesized from less expensive commercially available starting materials.

4-Chloro-7*H*-pyrrolo[2,3-d] pyrimidine was synthesized from cheap starting materials[29,30] (Scheme 2.1). Thus, the reaction of bromoacetal **1** with ethyl cyanoacetate (**2**) afforded ethyl diethoxyethylcyanoacetate **3** in 30 % yield after purification by vacuum distillation. Condensation of the mononitrile **3** with an ethanolic solution of

thiourea and sodium ethoxide furnished in 80 % yield the mercapto-pyrimidine **4** that was then subjected to a desulphurization reaction using Raney nickel to afford the corresponding pyrimidine **5** in 90 % yield. The pyrrolo-pyrimidone **6** was obtained in 65 % yield by spontaneous cyclisation of the acetal **5** when treated with hydrochloric acid at room temperature. Treatment of 4-hydroxypyrrolo pyrimidine **6** with phosphoryl chloride afforded the pure 4-chloro-7*H*-pyrrolo[2,3-d]pyrimidine (**7**) in 40 % yield after recrystallisation from ethyl acetate.

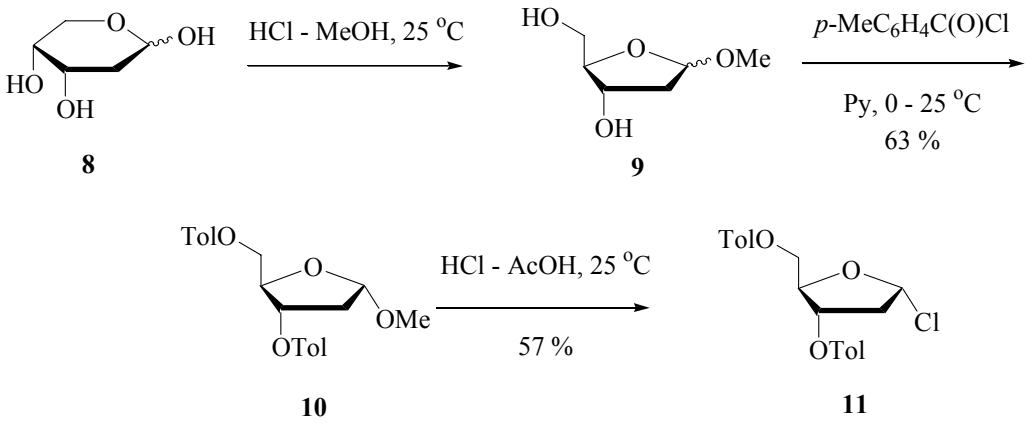
Scheme 2.1. Synthesis of 4-Chloro-7*H*-pyrrolo[2,3-d]pyrimidine



The sugar moiety, 2-deoxy-3,5-ditoluoyl pentafuranosyl chloride, **11**, has been synthesized from the accessible, commercially available 2-deoxy-ribose **8** according to a literature procedure (Scheme 2.2).[31,32] Thus, the 2-deoxy-ribose **8** was converted to the anomeric mixture of methyl pentofuranosides **9** using methanolic hydrogen chloride generated in situ by adding acetyl chloride to methanol. After the methanol was

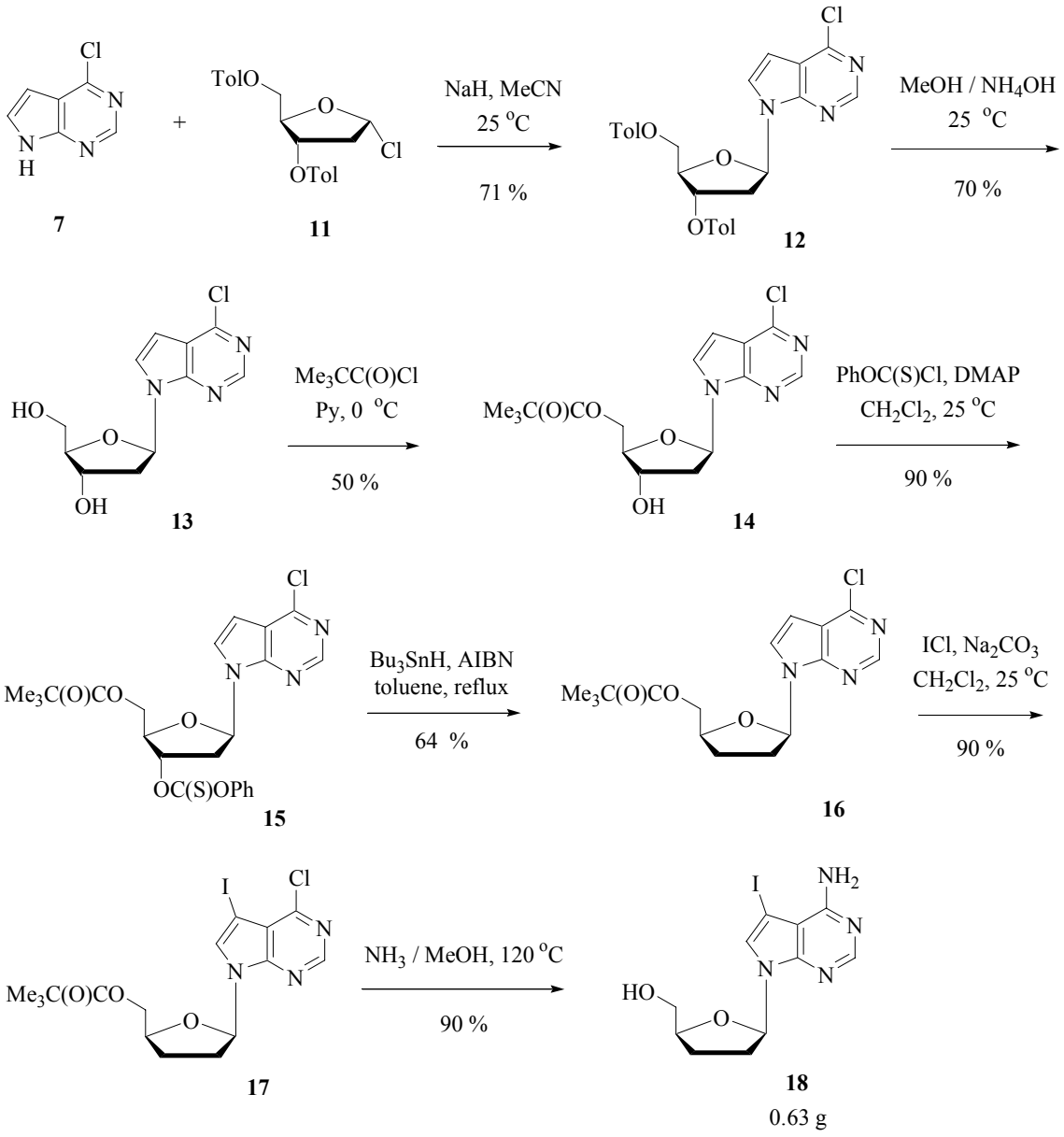
removed completely, the mixture was acylated by treatment with *p*-toluoyl chloride in pyridine to furnish a crude mixture of bis(4-methyl benzoates) **10**, which was purified by flash chromatography to give the α-isomer in 63 % yield. The α-methoxy derivative **10** was converted to the corresponding *α*-chloride by adding a saturated solution of hydrochloric acid in acetic acid prepared according to the same procedure used in the first step. The chloride precipitated immediately and afforded the *α*-chloride derivative in 57 % yield. This compound is stable in the solid state and decomposes when exposed to atmospheric humidity.

Scheme 2.2. Synthesis of 2-Deoxy-3,5-ditoluoyl pentafuranosyl Chloride



With the sugar and base parts in hand, we proceeded with the synthesis of the nucleoside, which was executed in 7 steps (Scheme 2.3).[26,28] Alkylation of the sodium salt of the deazapurine **7**, produced in situ by sodium hydride in acetonitrile, with 1-chloro-2-deoxy-3,5-ditoluoyl pentafuranose **11** afforded *β*-deoxiribonucleoside **12** in 71 % yield. Complete removal of the toluoyl groups was accomplished by extended treatment of the protected nucleoside with methanolic ammonia to furnish the diol **13** in 70 % yield. Regioselective protection of 5′-hydroxyl group with pivaloyl chloride gave the pivaloyl derivative **14** in 50 % yield. Compound 14 was then acylated with phenyl chlorothionocarbonate affording **15** in 90 % yield.

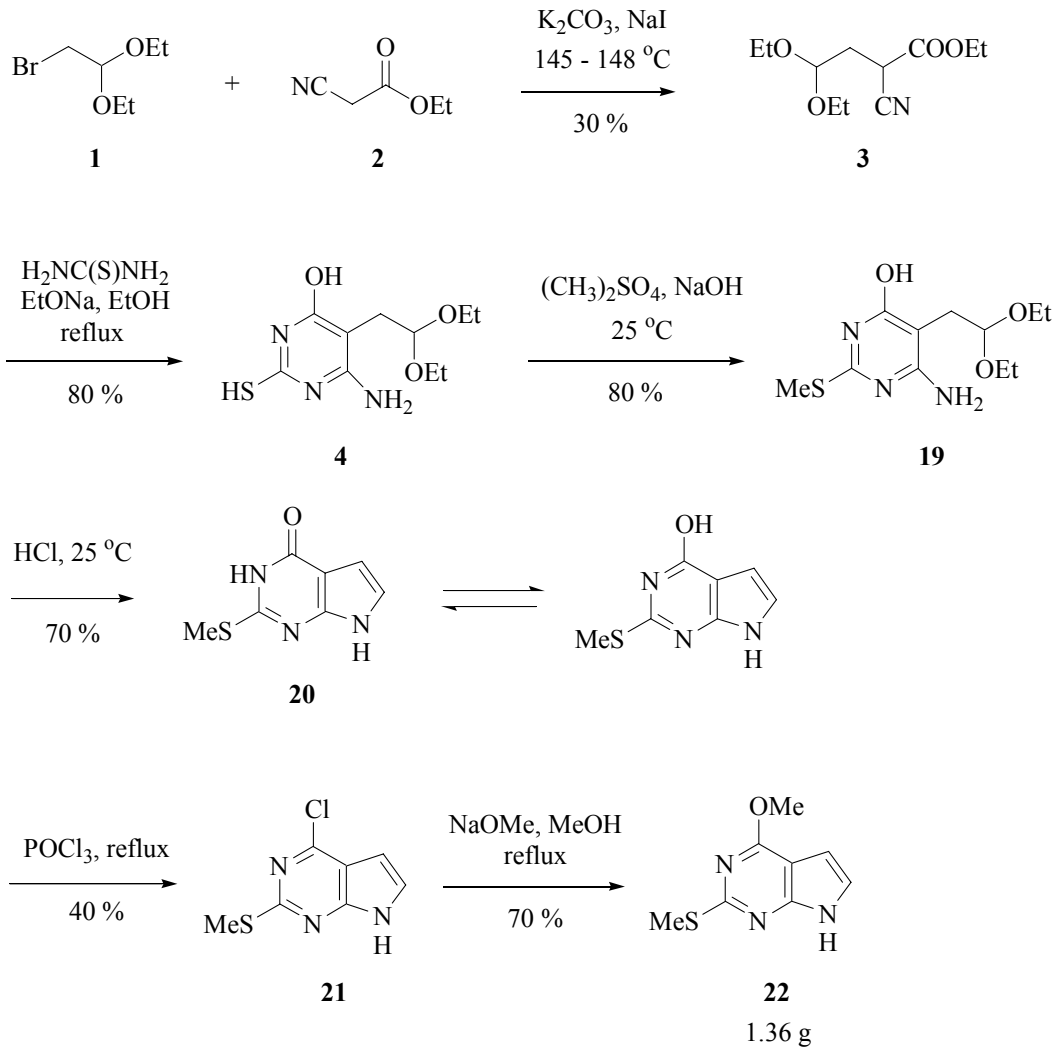
Scheme 2.3. Synthesis of 7-Iodo-2′,3′-dideoxy-7-deazaadenosine



Subsequent treatment of the latter with tributyltin hydride employed in Barton deoxygenation gave the corresponding dideoxynucleoside **16** in 64 % yield. The pivaloyl derivative of the dideoxynucleoside was then treated with an electrophilic reagent, iodine monochloride, and 7-iodo-7-deazapurine derivative **17** was isolated as

the only regioisomer in 90 % yield. Treatment of the latter with methanolic ammonia at 120 ^{o}C resulted in removing the pivaloyl protecting group with concomitant nucleophilic displacement of the 4-chloro group affording the target compound, 7-iodo-2′,3′-dideoxy-7-deazaadenosine, **18**, in 90 % yield. This compound was further used for converting into fluorescence-labeled chain terminating substrates for DNA polymerase.

Scheme 2.4. Synthesis of 4-Methoxy-2-methylsulfanyl-7*H*-pyrrolo [2,3-d]pyrimidine



In addition to 7-iodo-2′,3′-dideoxy-7-deazaadenosine, we also synthesized a common intermediate for the preparation of 7-iodo-2′,3′-dideoxy-7-deazaguanosine, 4-methoxy-2-methylsulfanyl-7*H*-pyrrolo[2,3-d]pyrimidine. It contains a bulky substituent at C-2 and its methoxy group avoids keto-enol tautomerism at N-1/O-6. These precautions diminish the electrophilic attack of N-2 and N-6 during glycosylation.

4-Methoxy-2-methylsulfanyl-7*H*-pyrrolo[2,3-d]pyrimidine (**22**) was prepared from bromoacetal **1** and ethyl cyanoacetate (**2**) in a manner similar to one described for synthesis of 4-chloro-7*H*-pyrrolo[2,3-d] pyrimidine (Scheme 2.4).[29,30,33] The mercapto-pyrimidine **4** was treated with dimethyl sulfate to afford 2-methylthiopyrimidine **19** in 80 % yield. Spontaneous cyclisation of the acetal **19**, which proceeded in 70 % yield, was followed by treatment with phosphoryl chloride affording the 4-chloro-derivative **21** in 40 % yield. Substitution of the chloro-group by a methoxy group using a solution of sodium methoxide in methanol furnished 4-methoxy-2-methylsulfanyl-7*H*-pyrrolo[2,3-d]pyrimidine (**22**) in 70 % yield. This compound can be further used for preparation of another precious nucleoside analogue, 7-iodo-2′,3′-dideoxy-7-deazaguanosine.

2.3 Conclusion

7-Iodo-2′,3′-dideoxy-7-deazaadenosine has been synthesized in 15 steps from affordable starting materials by a series of protection, deoxygenation, and iodination steps. 4-Methoxy-2-methylsulfanyl-7*H*-pyrrolo[2,3-d]pyrimidine, a common intermediate for the preparation of 7-iodo-2′,3′-dideoxy-7-deazaguanosine, has also been synthesized in 6 steps. Triphosphorylation of the 7-iodo-2′,3′-dideoxy-7-deazaadenosine, coupling the fluorescent dyes to the resulting nucleotide, its enzymatic incorporation into a growing DNA strand, and evaluation as chain-terminating inhibitors of the DNA polymerases are underway.

CHAPTER III

FLUORESCEIN-BASED DONOR COMPONENTS

3.1 Introduction

Fluorescein is one of the most common dyes used for fluorescence detection. It has a large fluorescence quantum yield (0.92 at pH > 8), good water solubility, a favorable absorption spectrum, and it is commercially available in many derivatives. In addition, fluorescein has an absorption maximum (494 nm) that closely matches the 488 nm spectral line of the argon lasers employed by the two most widely used DNA sequencers, Applied Biosystems 3700 and 3730 DNA sequencers.

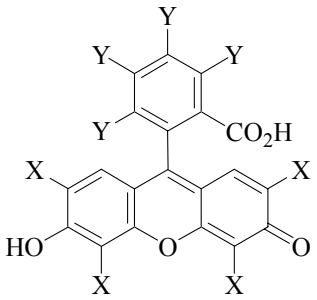
Owing to the advantages that fluorescein possesses, fluorescein derivatives are widely used as fluorescent detection reagents in many biochemical experiments to label macromolecules, and for this reason, over the years, a variety of functionalized fluoresceins have been prepared. However, in spite of their widespread use in biotechnology, fluorescein-based components and their conjugates have well-known deficiencies. Fluorescein conjugates are unstable - they photobleach too easily, resulting in a fast decrease in fluorescent signal.[34] Other drawbacks are that the absorption and fluorescence properties of fluorescein are pH dependent and fluorescein-based dyes have a tendency toward quenching of their fluorescence when conjugating to biomolecules. In those biochemical experiments where sensitivity is critical (e.g. when preparing conjugates of the dyes to proteins), the fluorescein-based donors are replaced by different donors (e.g. rhodamines, fluorinated fluoresceins[34]).

The synthesis of fluorescein was first reported in 1871 by Von Bayer.[35] It was easily prepared by the condensation of resorcinol with phthalic anhydride in the presence of zinc chloride as a catalyst. However, preparation of fluorescein derivatives using similar methods with substituted phthalic anhydrides is difficult because the reaction gives mixtures of regioisomers and isolation of the pure products proved to be a serious problem. Halogenated fluoresceins are useful substrates in Suzuki and Sonogashira

coupling reactions. The Sonogashira coupling[36] is widely used in the synthesis of fluorescent donor-acceptor dyes. Coupling of aryl halides with a monosubstituted alkyne to obtain a 1,2-disubstituted acetylene is accomplished by palladium(0) and a co-catalyst, copper iodide.

Methodologies for preparation of pure regioisomers of halogenated fluoresceins have been developed and the derivatives mentioned below are just a few examples that illustrate this. It is necessary to prepare isomerically pure fluoresceins because multistep preparation of fluorescein derivatives requires pure regioisomers in order to purify and identify easily the reaction products. On the other hand, when mixtures of 5- and 6-isomers are conjugated to biomolecules, there are differences in conjugate polarity and internal fluorescence quenching.[37] Thus, 2′,4′,5′,7′-tetrabromofluorescein (eosin **1**), 2′,4′,5′,7′-*tetra*-iodofluorescein (erythrosine **2**), and 2′,4′,5′,7′- *tetra*-bromo-4,5,6,7-*tetra*-chlorofluorescein (rose bengal **3**) shown in Figure 3.1 were prepared right after fluorescein was originally synthesized, but they suffer from much lower fluorescence quantum yields compared with fluorescein. Fluorescein, which is unsubstituted, has a high fluorescence quantum yield, while Eosin, Erythrosin, and Rose Bengal intersystem-cross to the triplet state and have a higher population of triplet relative to singlet, fluorescing with lower quantum efficiency.[38] Heavy atoms substitution in an aromatic molecule usually increases singlet-triplet transitions owing to increased spin-orbit mixing of the singlet and triplet manifolds. Substitution by heavy atoms may affect the vibrational state and change the fluorescence spectra, and the electronic excitation energy is converted into vibrational energy, the fluorescence becoming weak.[39] For example, the four iodine atoms of Rose Bengal increase its intersystem-crossing yield relative to fluorescein, which means that Rose Bengal fluoresces red, but weakly. On halogen substitution, a decrease in fluorescence quantum yield is observed in the sequence of Cl, Br, and I owing to the heavy atom effect. Unlike these halogenated fluoresceins, which have expected properties resulting from the substitution effect, the substitution of hydrogen atoms by fluorine atoms produce intense changes in their properties, mainly owing to the highly electronegative nature and small Van der Waals

radius of the fluorine atom. Regioisomerically pure fluorinated fluoresceins have been synthesized. They proved to be superior to other fluorescein derivatives for bioconjugation owing to their high quantum yield, resistance to photobleaching, and decreased quenching when conjugating to biomolecules.[34]



1: X = Br, Y = H
2: X = I, Y = H
3: X = I, Y = Cl

Figure 3.1. Fluorescein derivatives.[34]

Preparation routes to pure regioisomers of 5- or 6-halofluoresceins have been reported as well. They are prepared by condensation of resorcinol with substituted phthalic anhydride and sulfocarboxy anhydride, respectively.[40] They are valuable substrates for preparation of the fluorescein-based donor components (Figure 3.2) to be incorporated into through-bond energy transfer cassettes for DNA sequencing and other applications in biotechnology.[16] Because they are important synthetic building blocks used to make various dyes, our efforts have been focused on making more fluorescein-based donors via halogenated fluoresceins. This chapter describes synthesis of pure halofluoresceins and a fluorescein-based donor component, 5-ethynylfluorescein-(5-*tert*-butoxycarbonyl)-pentyl ester.

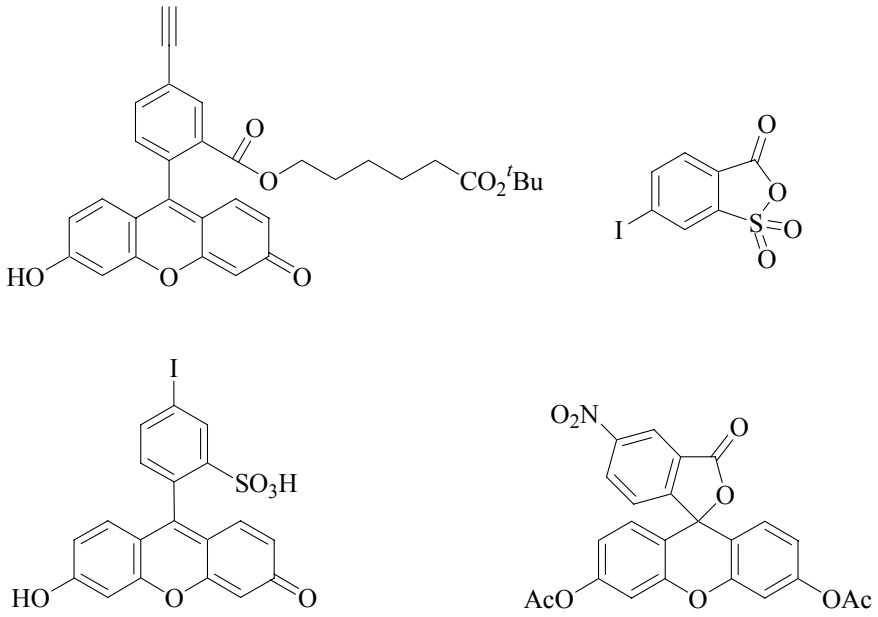


Figure 3.2. Target compounds for Chapter III.

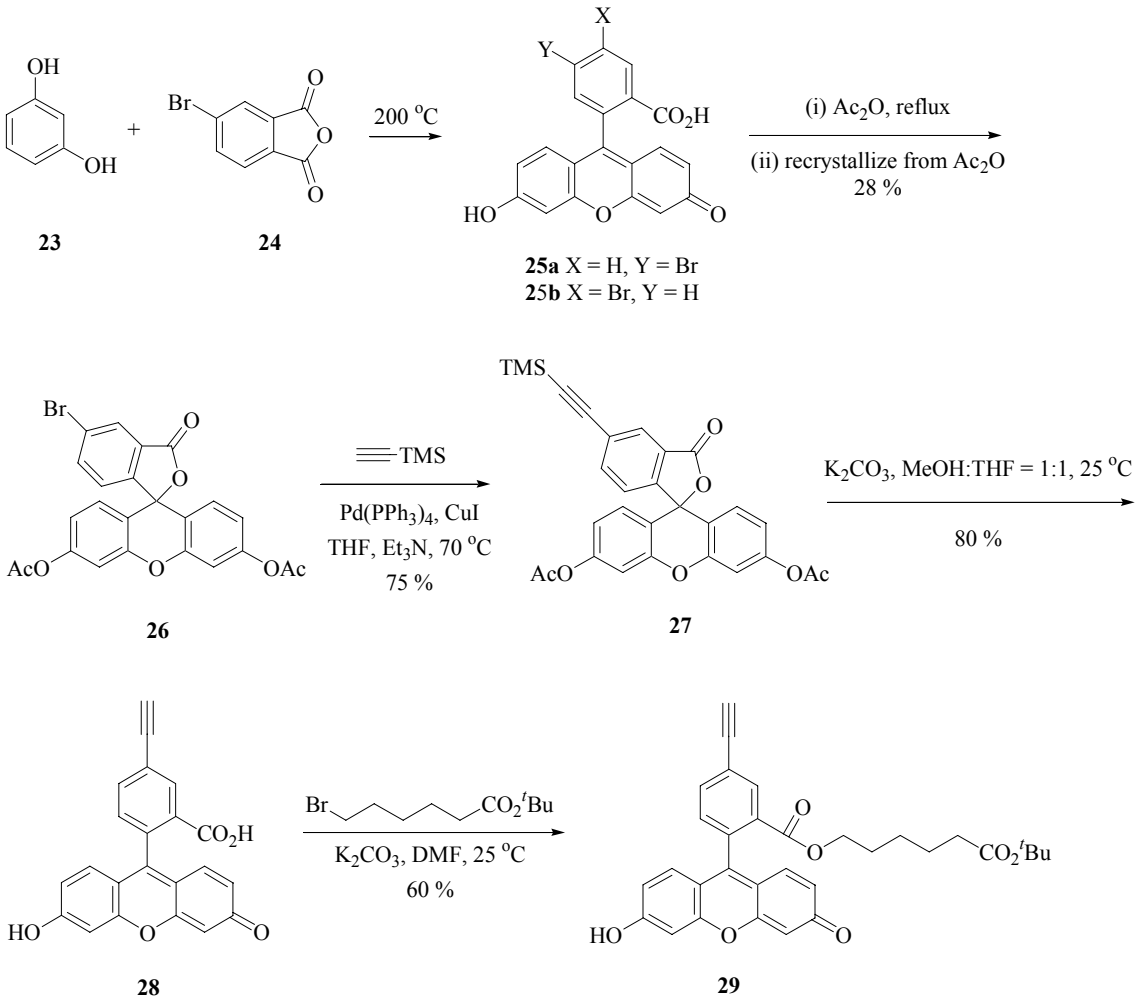
3.2 Synthesis of 5-Ethynylfluorescein-(5-*tert*-butoxycarbonyl)-pentyl Ester

5-Ethynylfluorescein-(5-*tert*-butoxycarbonyl)-pentyl ester (**29**) is a fluorescein-based donor fragment useful for incorporation into through-bond energy transfer dyes designed especially for DNA sequencing. It contains two significant functionalities - one is used to attach the acceptor fragment, and the other one is used to add the linker for attachment to biomolecules. The synthesis of 5-ethynylfluorescein-(5-*tert*-butoxycarbonyl)-pentyl ester (**29**) was executed in 5 steps (Scheme 3.1) starting with accessible starting materials according to a procedure reported by Jiao *et. al*.[40] and by Thoresen *et. al*.[41] Thus, condensation of 1,3-dihydroxybenzene (**23**) with 4-bromo phthalic anhydride (**24**) yielded 5- and 6-bromofluoresceins **25a** and **25b** as a mixture of two regioisomers. The crude mixture of these two isomers was subjected to acylation reaction with acetic anhydride without further purification. 5-Bromofluorescein was isolated as the ring closed diacetate by fractional crystallization from acetic anhydride. The reaction yield in 5-bromofluorescein diacetate was 28 % and no chromatography was necessary. The 6-isomer was also isolated as the ring-closed diacetate from the

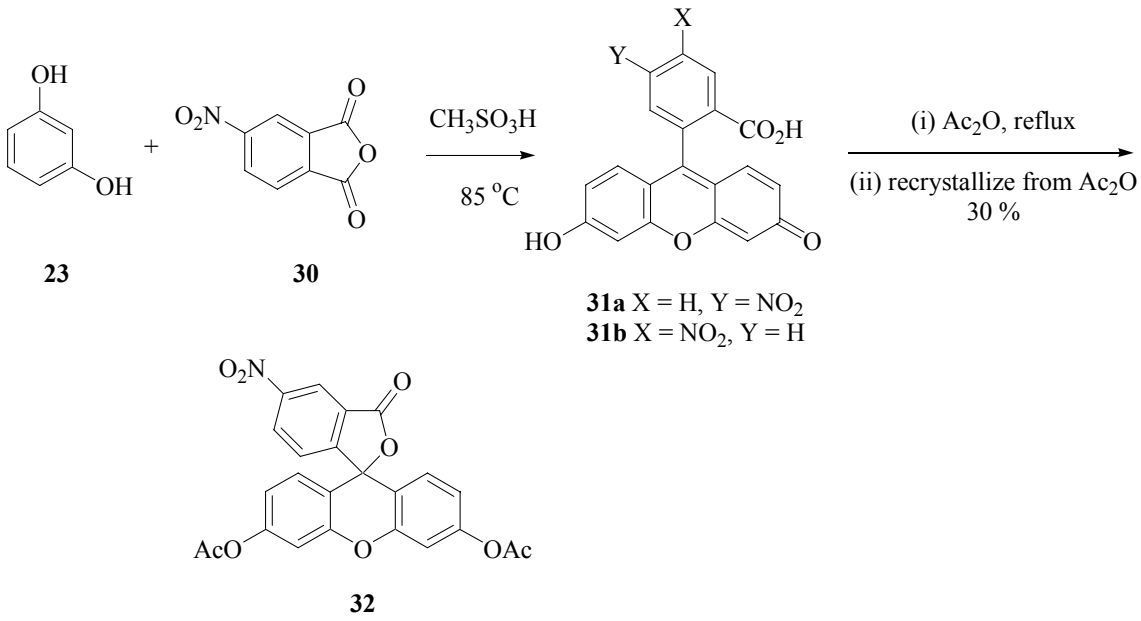
mother liquor by repeated recrystallization from ethanol. 5-Bromofluorescein diacetate was subjected to Sonogashira reaction using trimethylsilyl acetylene in the presence of Pd(0) catalyst to afford 5-(2-trimethylsilylethynyl) fluorescein diacetate **27** in 75 % yield after recrystallization from ethanol. Deprotection of **27** with potassium carbonate gave in 80 % yield 5-ethynyl fluorescein **28** that precipitates from the reaction conditions when acidified with concentrated HCl. A linker for attachment to biomolecules was installed by alkylating the carboxylate of 5-ethynylfluorescein with the *tert*-butyl ester of 6-bromohexanonoic acid to furnish in 60 % yield the target compound, 5-ethynylfluorescein-(5-*tert*-butoxycarbonyl)-pentyl ester **29** used further for incorporation into through-bond energy transfer cassettes.

In the context of halogenated fluoresceins, in addition to bromofluoresceins, other valuable substrates used for preparation of fluorescein-based donors are iodofluoresceins. They can be prepared via diazotization/iodination of the corresponding aminofluoresceins that are commercially available. However, owing to their high cost, aminofluoresceins were synthesized from affordable starting materials via the same route as the one used for preparation of bromofluoresceins according to the literature procedure (Scheme 3.2).[42,43] Thus, condensation of 1,3-dihydroxybenzene (**23**) with 4-nitrophthalic anhydride (**30**) gave a mixture of 5- and 6-nitrofluoresceins **31a** and **31b**, which was further used for acylation to the ring-closed diacetate. 5-Nitrofluorescein diacetate **32** was isolated, as the only isomer, via fractional crystallization from acetic anhydride in 30 % yield. Further reduction of the nitro group, followed by diazotization and treatment with potassium iodide afford the corresponding iodofluorescein, an important intermediate for preparation of fluorescein-based donors to be incorporated into donor-acceptor cassettes.

Scheme 3.1. Synthesis of 5-Ethynylfluorescein-(5-*tert*-butoxycarbonyl)-pentyl Ester



Scheme 3.2. Synthesis of 5-Nitrofluorescein Diacetate



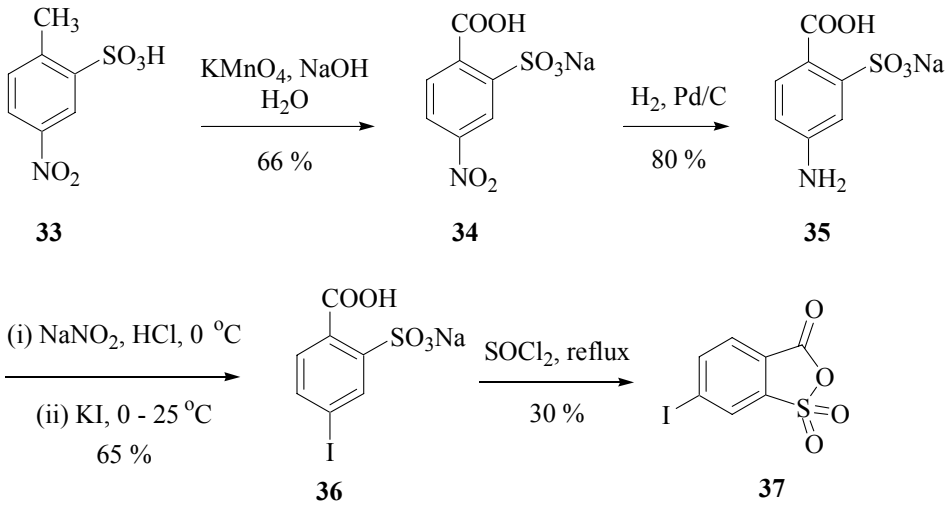
3.3 Synthesis of 5-Iodosulfofluorescein

Other useful halofluoresceins used in preparation of fluorescent donor-acceptor cassettes are sulfonic acid derivatives of 5-iodofluoresceins. Compared to fluoresceins, the sulfonic acid derivatives enjoy greater water solubility as they contain the sulfonic acid group. Moreover, only one isomer can form when using substituted 2-sulfobenzoic anhydrides as substrates for preparation of sulfofluoresceins since only one carbonyl group is available for condensation. This is a significant advantage of sulfofluoresceins since the laborious separation of regioisomers is not necessary. Described here are the preparation of sulfocarboxy anhydride and its condensation with 1,3-dihydroxybenzene to form sulfofluoresceins according to the literature procedure.[44, 45]

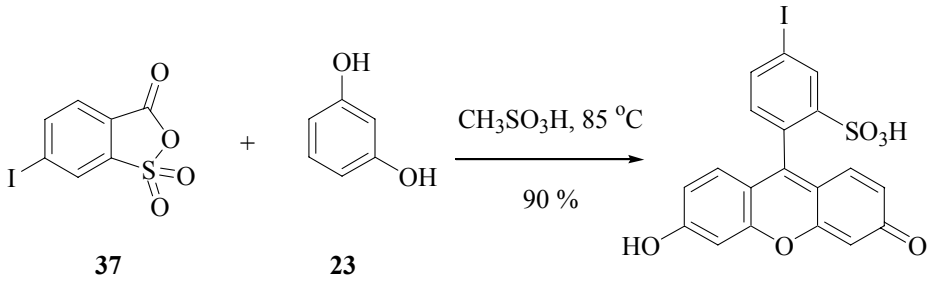
Synthesis of 5-iodo-2-sulfobenzoic anhydride was executed in 5 steps starting from accessible starting materials according to the literature procedures (Scheme 3.3).[44-46] Thus, oxidation of 2-methyl-5-nitrobenzenesulfonic acid (**32**) with potassium permanganate afforded **33** in 66 % yield. The sodium salt **33** was then subjected to a

hydrogenation reaction with palladium catalyst to furnish the aminoderivative **35** in 80 % yield. Diazotization, followed by iodination of the corresponding aminoderivative afforded **36** in 65 % yield. This was treated then with thionyl chloride to furnish 5-iodo-2-sulfobenzoic anhydride **37** in 30 % yield. Condensation of sulfocarboxy anhydride **37** with 1,3-dihydroxybenzene (**23**) in the the presence of methanesulfonic acid which acts both as solvent and catalyst, yielded the target compound **38**, a 5-iodosulfofluorescein derivative, in 90 % yield (Scheme 3.4)**.** The product precipitated from the reaction conditions when a limited quantity of water was added to the reaction mixture.

Scheme 3.3 Synthesis of 5-Iodo-2-sulfobenzoic Anhydride



Scheme 3.4. Synthesis of 5-Iodosulfofluorescein Derivative



37 **23** **38**

0.038 g

3.4 Conclusion

A fluorescein-based donor component, 5-ethynylfluorescein-(5-*tert*-butoxycarbonyl)-pentyl ester, has been prepared in five steps starting with accessible starting materials, 1,3-dihydroxybenzene and 4-nitrophthalic anhydride, via 5-bromofluorescein diacetate. The pure regioiosmer of bromofluorescein diacetate has been isolated by repeated fractional crystallization from acetic anhydride and can be prepared in gram quantities.

In the context of halofluoresceins, pure 5-nitrofluorecein diacetate has been prepared and isolated via the same route as the one described for bromofluorecein. Further reduction/diazotization/iodination reactions give the corresponding iodofluoresceins, important intermediates for preparation of fluorescein-based donors to be incorporated into donor-acceptor cassettes.

Preparation of sulfonic acid derivatives enjoys the advantage that only one isomer can form when using substituted 2-sulfobenzoic anhydrides since only one carbonyl group is available for condensation. This way the laborious separation of regioisomers is not necessary. Synthesis of 5-iodo-2-sulfobenzoic anhydride was executed in 5 steps starting from accessible starting materials.

Fluorescein-based donor fragments are important synthetic building blocks to be incorporated into through-bond energy transfer cassettes for DNA sequencing and other applications in biotechnology.

CHAPTER IV

RHODAMINE-BASED ACCEPTOR COMPONENTS

4.1 Introduction

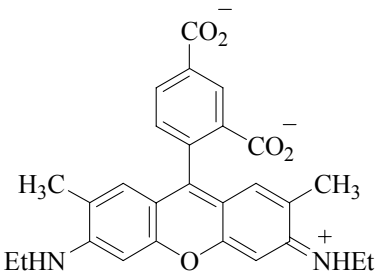
Rhodamine dyes are important owing to their favorable photochemical and photophysical properties. Thus, rhodamine dyes have many favorable fluorescence properties – they have relatively long absorption maxima, they have high quantum yields (0.45 to 0.95) and high molar extinction coefficients. Compared to the fluorescein dyes, rhodamine dyes are relatively resistant to photobleaching and their fluorescence spectra are pH-independent over a wider pH range (from 4 to 10).[47] Moreover, dye-terminators labeled with rhodamine dyes are more efficiently incorporated by DNA polymerase *Taq*FS.[9]

Because of their high fluorescence quantum yield and photostability, rhodamine dyes have widespread applications, not only in biotechnology, e.g. fluorescent labeling, single molecule detection, etc., but also in medicine for staining damaged cells, etc.

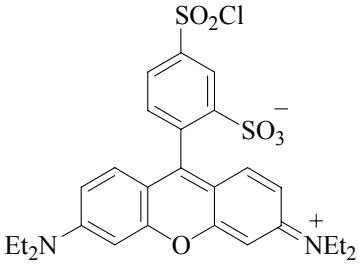
The ideal rhodamine-based acceptors for the energy transfer systems useful for cellular imaging should emit above 650 nm. However, rhodamines have emission wavelengths shorter than ideal, so that rhodamines with extended and planar aromatic systems are of more interest. The rhodamine dyes that emit at wavelengths above 650 nm avoid background signals caused by biological autofluorescence.

Various rhodamine dyes have been prepared and used as fluorescent labeling reagents over the years. 5-Carboxyrhodamine 6G hydrochloride, lissamine rhodamine B sulfonyl chloride, Texas Red sulfonyl chloride, and tetramethylrhodamine isothiocyanate are among the most widely used fluorescent reagents. Their structures are shown in Figure 4.1. Texas Red derivatives, that contain julolidine rings, enjoy an increase in their fluorescence quantum yields relative to those of the rhodamine derivatives without extended aromatic systems. This increase is due to the fact that these rings prevent

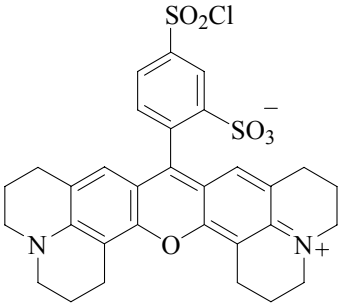
rotation about the nitrogen atoms and lead to a shift of their fluorescence spectra to longer wavelengths.



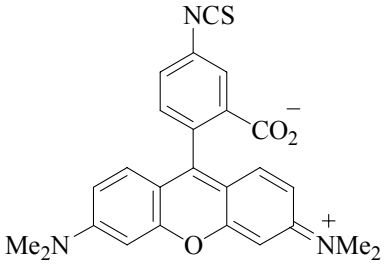
5-carboxyrhodamine 6G hydrochloride



lissamine rhodamine B sulfonyl chloride



Texas Red sulfonyl chloride

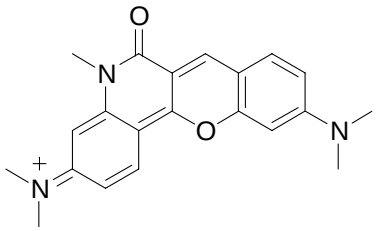


tetramethylrhodamine isothiocyanate

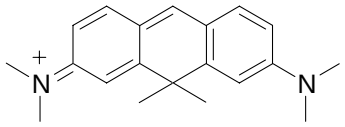
Figure 4.1. Representative structures of rhodamine derivatives.[48]

Two other new classes of dyes that intensely fluoresce in the 600–700 nm range are shown in Figure 4.2. Their strong fluorescence efficiency is due to the planarity and rigidity of the major part of the systems. The first class, the amide-bridged benzopyrylium dyes, are related to rhodamines, while the second class, carbopyronin dyes, are derived from rhodamines by replacing the O-atom by a carbon atom, for both classes their absorption and emission spectra are shifted to longer wavelengths. These

types of dyes might be varied in many ways to obtain halogenated acceptor components used as fluorescent labels.



amidopyrylium dye



carbopyronin dye

Figure 4.2. Amide-bridged benzopyrylium and carbopyronin dyes.[49,50]

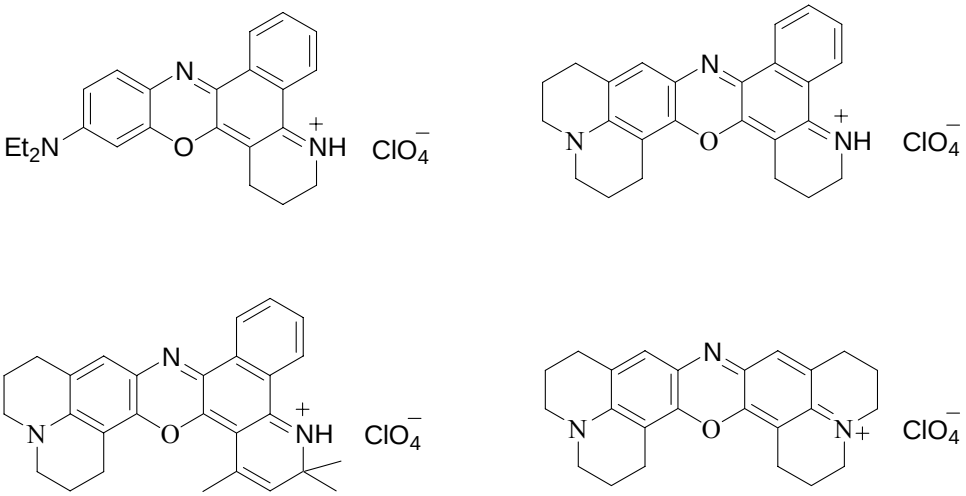


Figure 4.3. Bridged naphthoxazinium salts.[51]

Bridged naphthoxazinium salts are another type of extended rhodamine derivatives with emission maxima above 640 nm. A few examples of this class of compounds are shown in Figure 4.3. Their absorption wavelengths range from 605 to 670 nm and their emission maxima vary between 645 and 712 nm. These bridged naphthoxazinium salts exhibit moderate quantum yields and their absorption and

emission properties are dependent on the polarity and pH of the solvents used. Consequently, they can also be used as acid-base indicators.

Bromo-substituted rhodamine derivatives are very useful starting materials for preparation of fluorescent dyes via coupling reactions. A set of four bromo-rhodamines is shown in Figure 4.4. They have favorable fluorescence spectra that range from 532 to 616 nm and their synthesis is convenient and efficient in the context of obtaining pure regioisomers. Consequently, they are useful synthetic building blocks and have been used as acceptor components and incorporated into through-bond energy transfer systems via both Sonogashira and Suzuki organometallic coupling reactions with fluorescein derivatives.[16,52] When these fluorescent systems have been excited by a laser source that operates at a single wavelength (e.g. 488 nm, the excitation from an Ar-laser), they fluoresce at wavelengths characteristic of only the acceptor components.

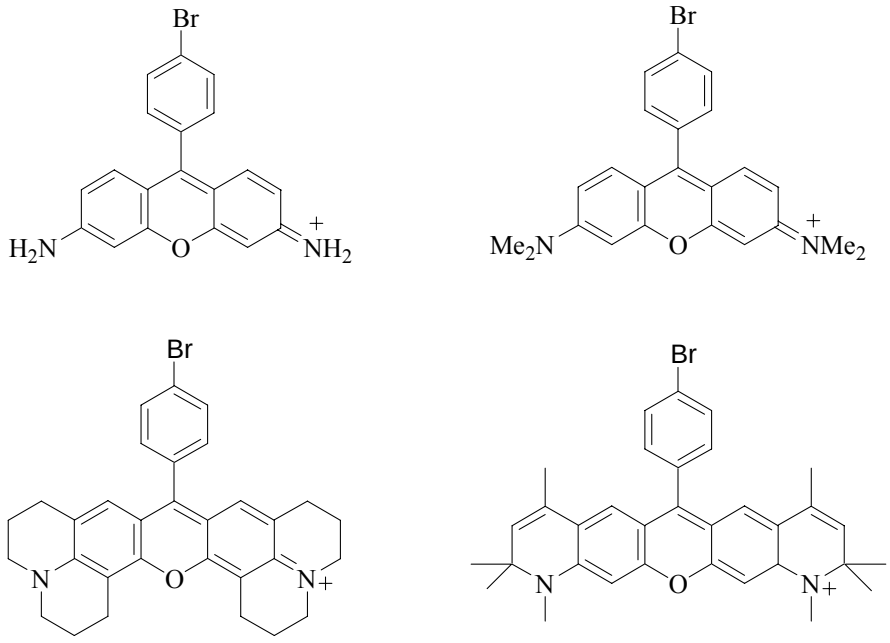


Figure 4.4. Bromo-substituted rhodamine derivatives.[53]

The rhodamine-based acceptor components described later in this chapter are designed and synthesized mainly for intracellular imaging. Consequently, they should emit with high quantum yields above 650 nm in order to minimize the problems of background fluorescence from the components in cells. Since there are very few fluorescent dyes that are consistent with biological samples and emit efficiently above 650 nm, the design and synthesis of acceptor components that produce long wavelength emission is needed. Although rhodamines have been established to be superior to other fluorophores with respect to photostability and long wavelength emission maxima, they are not appropriate for intracellular studies since their emission wavelengths are shorter than ideal.[48] The extended rhodamine derivatives mentioned earlier emit in the range 640–700 nm and could be converted into the corresponding halogenated acceptor components, so that they might act as potential acceptor fragments for the through-bond energy transfer system targets. However, modified rhodamine-based acceptors with extended aromatic systems whose synthesis is not that laborious need to be prepared. Types of these modified rhodamines are pyrylium cations that are expected to fluoresce above 650 nm. Similar pyrylium cations without amino groups were prepared for other reasons before and are known to be highly fluorescent.[54-57] One of the target cassettes has been designated to have a rhodamine-based donor with one sulfonic acid group to improve water solubility coupled with the above designed rhodamine-based acceptor with carboxylic acid functionalities for attachment of the dyes to biomolecules (Figure 4.5). The synthesis of the rhodamine-based acceptor is described later in detail in the next section of this chapter.

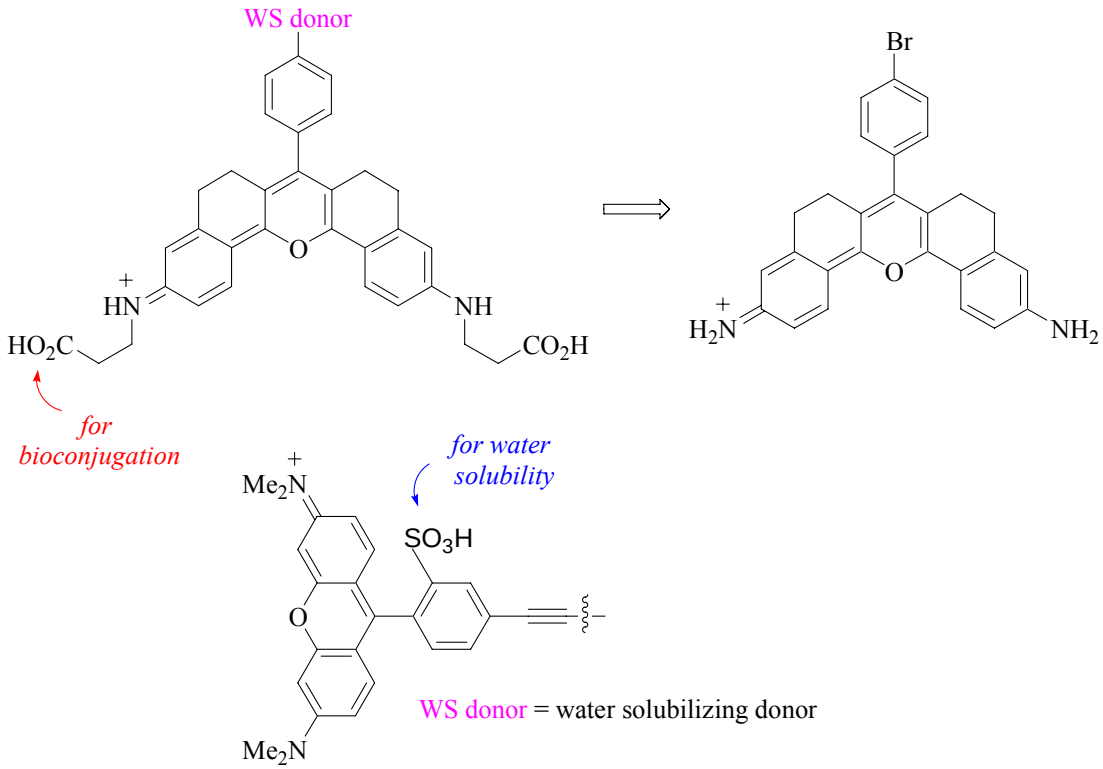


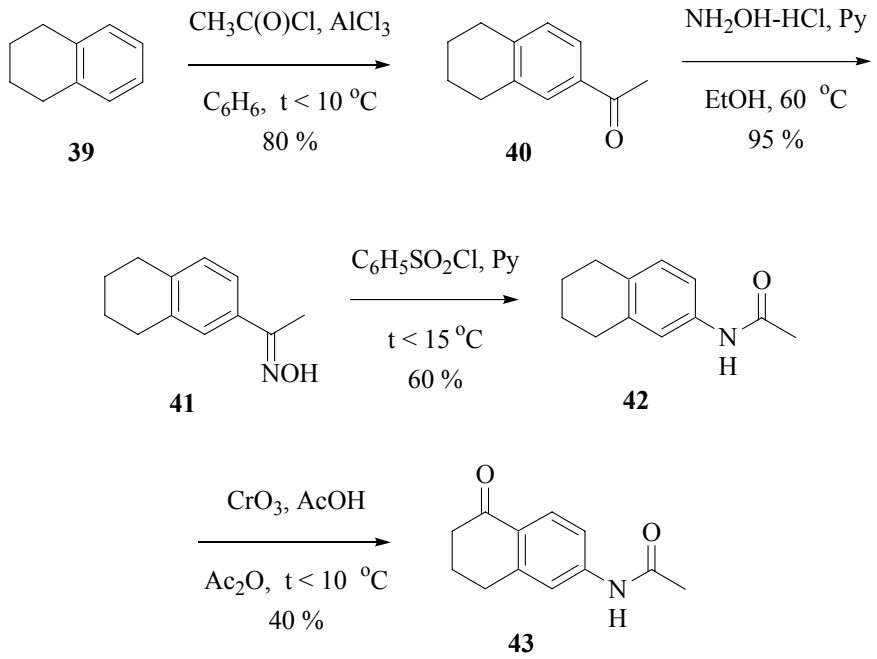
Figure 4.5. Target cassette optimized for maximal fluorescence intensity.

4.2 Synthesis of Pyrylium Cations

The rhodamine-based acceptor fragments that I synthesized are pyrylium cations and the starting materials chosen to start their synthesis are 6-acetylaminotetralone and 2-benzylidene-1-tetralone. Synthesis of 6-acetylaminotetralone **43** was executed in four steps from a cheap commercially available starting material, tetralin (**39**), according to literature procedures (Scheme 4.1).[58-61] Thus, Friedel-Crafts acetylation of tetralin (**39**) with acetyl chloride in the presence of aluminum chloride furnished acetyltetralin **40** in 80 % yield after purification by vacuum distillation. Condensation of the latter with hydroxylamine was performed according to a modified literature procedure and furnished the crystalline oxime **41** in 95 % after recrystallization from ethyl acetate. The oxime **41** was subjected to Beckmann rearrangement using benzenesulphonyl chloride in pyridine to furnish 6-acetamidotetralin **42** in 60 % yield. Oxidation of the resulting 6-

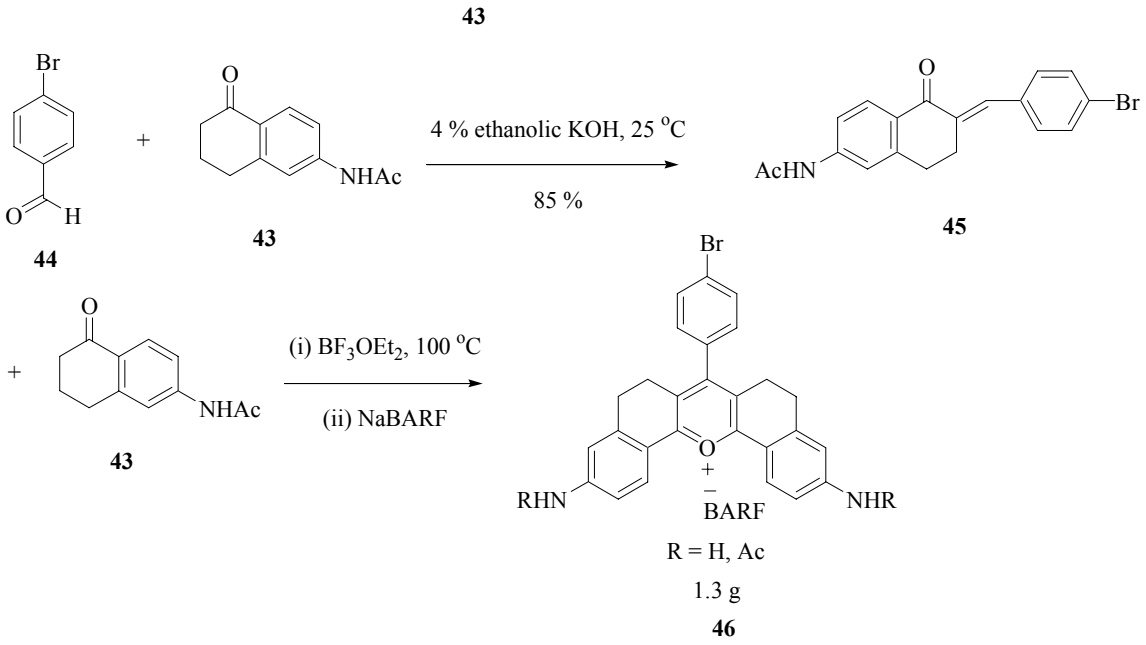
acetylaminotetralin with chromium trioxide in acetic acid afforded 6-acetamidotetralone **43** in 40 % yield.

Scheme 4.1. Synthesis of 6-Acetylaminotetralone



6-Acetamidotetralone **43** is a common intermediate for preparation of both 6-acetamido-2-benzylidene-1-tetralone and pyrylium cations. Preparation of diamonopyrylium salt **46** is shown in Scheme 4.2.[54,62] Thus, condensation of bromobenzaldehyde **44** with the readily prepared 6-acetamidotetralone **43** in ethanolic potassium hydroxide furnished crystalline 6-acetamido-2-benzylidene-1-tetralone (**45**) in 85 % yield after recrystallization from ethanol. 6-Acetamido-2-benzylidene-1-tetralone (**45**) was further subjected to a condensation reaction with 6-acetamidotetralone **43** in the presence of boron trifluoride-ether as a hydride abstracting agent to give the pyrylium tetrafluoroborate. Unfortunately, the condensation reaction yielded 1.3 g of mixture of diaminopyrylium salt, monoacetamido- and diacetamidopyrylium salts. A reaction time of four hours was not sufficient for complete condensation.

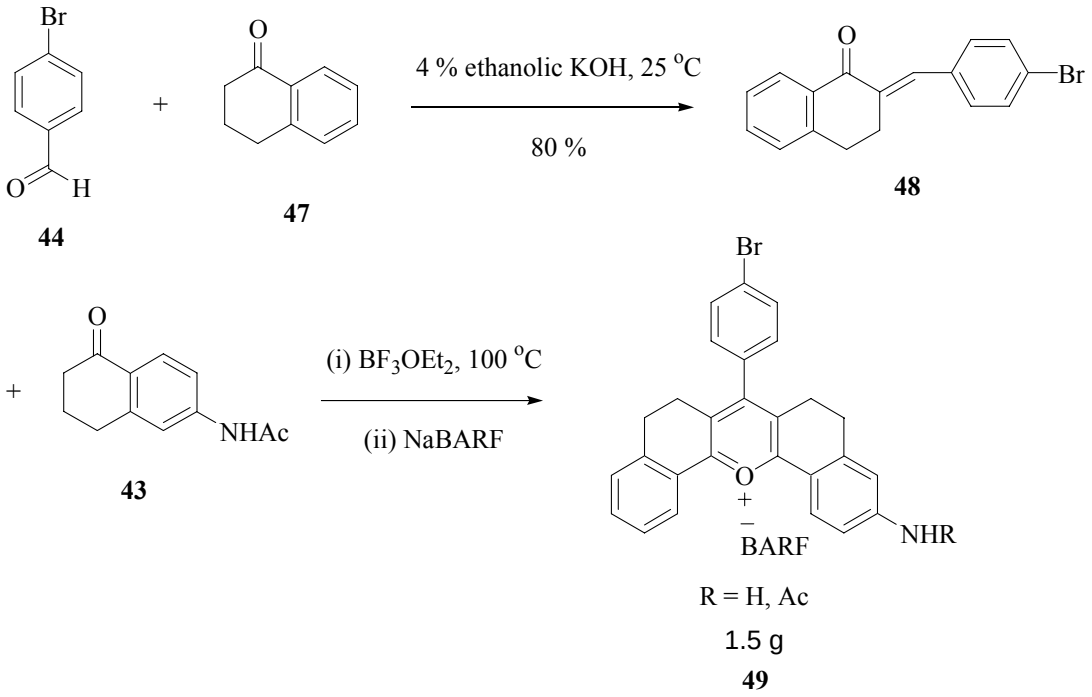
Scheme 4.2. Synthesis of Diaminopyrylium Cation



Consequently, the reaction was optimized by use of longer reaction times. However, after 24 hours, monoacetamido derivative was still present in the reaction mixture. The efforts to isolate the product by recrystallization from various solvent systems were unsuccessful, so that other approaches were taken. Thus, in the attempt to isolate diaminopyrylium salt, the counterion was exchanged with *tetra*(3,5-trifluoromethylbenzene)boronate (BARF) anion in order to allow convenient chromatographic isolation of the product. Unfortunately, the flash chromatography did not help isolate the diaminopyrylium salt either. HPLC could be used to purify the desired product, but its unavailability did not allow me further attempts to isolate it.

In addition to diaminopyrylium salt **46**, monoaminopyrylium salt **49** was prepared following the same procedure as for **46** (Scheme 4.3). Thus, condensation of bromobenzaldehyde **44** with α-tetralone **47** furnished the crystalline 2-benzylidene-1-tetralone **48** in 80 % yield. Further condensation of **48** of with 6-acetamidotetralone **43** afforded the monoaminopyrylium salt **49** that was subjected to the same isolation procedure as the one mentioned above for **46**.

Scheme 4.3. Synthesis of Monoaminopyrylium Cation



4.3 Conclusion

New rhodamine-based acceptor components with extended aromatic systems were synthesized. Types of these modified rhodamines are monoamino- and diaminopyrylium cations like **46** and **49**. They were prepared from affordable starting materials, tetralin and 4-bromo-benzaldehyde. Synthesis of the intermediate 6-acetylaminotetralone **43** was executed in four steps starting from tetralin. 2-Benzylidene-1-tetralones **45** and **48** were prepared by the condensation of 4-bromo-benzaldehyde with the corresponding tetralones and were then subjected to further condensations in the presence of boron trifluoride-ether to give the pyrylium salts. The efforts to isolate the pyrylium salts by repeated recrystallizations from various solvent systems and flash chromatography after exchanging the counterion with *tetra*(3,5-trifluoromethylbenzene)boronate to allow convenient chromatographic isolation of the product were unsuccessful. It should be possible to isolate the desired target compounds using HPLC. Once isolated, these rhodamine-based acceptors could be potential

components to be incorporated into through-bond energy transfer cassettes for intracellular imaging.

CHAPTER V

CONCLUSION

A nucleoside analogue, 7-iodo-2′,3′-dideoxy-7-deazaadenosine, has been synthesized in 15 steps from affordable starting materials by a series of protection, deoxygenation, and iodination steps. 4-Methoxy-2-methylsulfanyl-7*H*-pyrrolo[2,3-d]pyrimidine, a common intermediate for the preparation of 7-iodo-2′,3′-dideoxy-7-deazaguanosine, has also been synthesized in 6 steps. Triphosphorylation of the 7-iodo-2′,3′-dideoxy-7-deazaadenosine, coupling the fluorescent dyes to the resulting nucleotide, its enzymatic incorporation into a growing DNA strand, and evaluation as chain-terminating inhibitors of the DNA polymerases are underway (Figure 5.1).

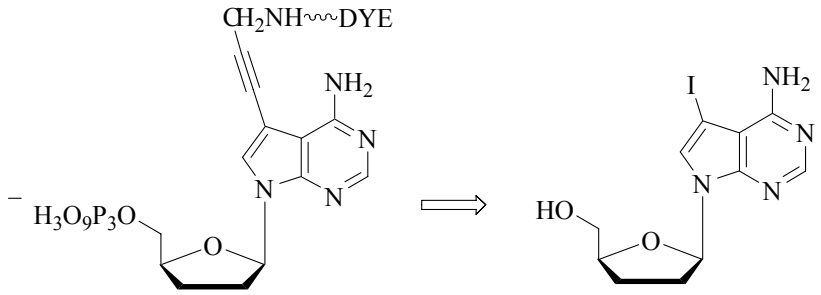


Figure 5.1. Chain-terminating inhibitor of the DNA polymerase.

Chapter III describes the synthesis of fluorescein-based donor components to be incorporated into through-bond energy transfer systems. Although they are known compounds, our efforts have been focused on making more fluorescein-based donors via halogenated fluoresceins since they are important synthetic building blocks used to make various dyes. The synthesis of fluorescein-based donor fragment has been executed in five steps from 1,3-dihydroxybenzene and phthalic anhydride. The donor fluorescein carboxylate has been alkylated with the *tert*-butyl ester of 6-bromohexanonoic acid to

provide a handle for attachment to biomolecules. In the context of regioisomerically pure halofluoresceins, besides the synthesis of pure regioisomers of bromofluorescein derivatives, 5-iodosulfofluorescein and pure regioisomers of 5-nitrofluorescein diacetate, as an intermediate in the synthesis of 5-iodofluoresceins, have been synthesized.

Chapter IV describes the synthesis of new rhodamine-based acceptor components with extended aromatic systems. Types of these modified rhodamines are monoamino- and diaminopyrylium cations like **46** and **49**. They were prepared from affordable starting materials, tetralin and 4-bromo-benzaldehyde. The efforts to isolate the pyrylium salts by repeated recrystallizations from various solvent systems and flash chromatography after exchanging the counterion with *tetra*(3,5-trifluoromethylbenzene)boronate (BARF) to allow convenient chromatographic isolation of the product were unsuccesssful.

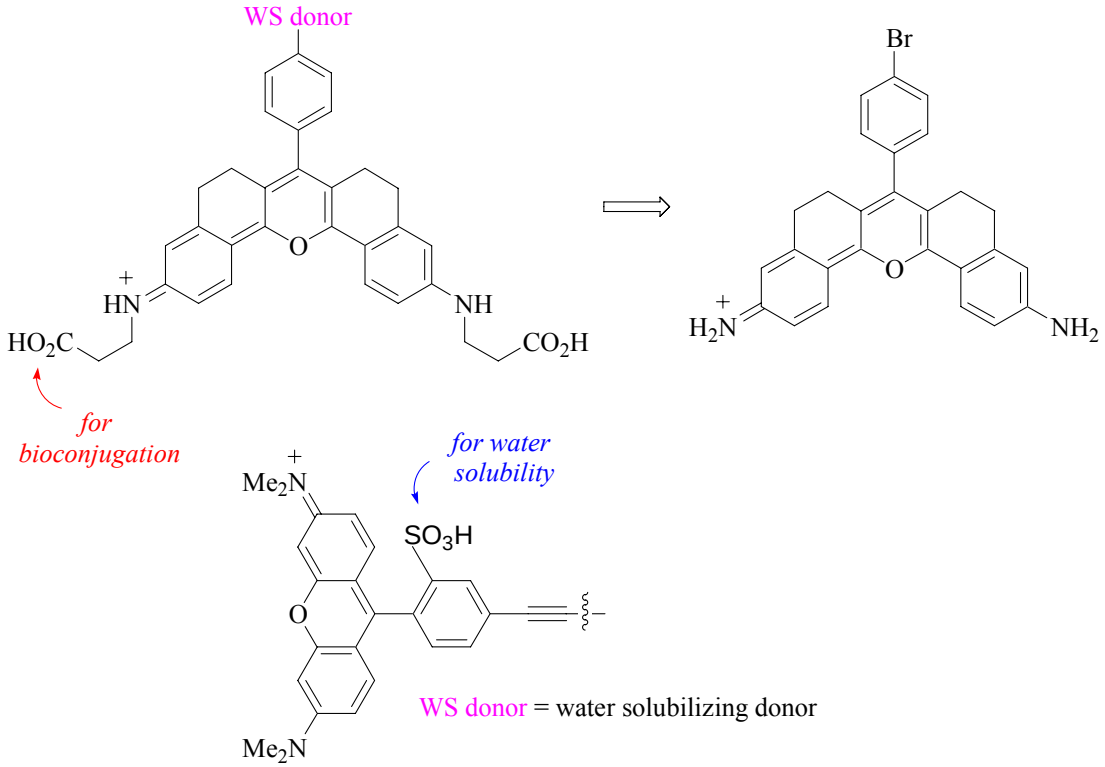


Figure 5.2. Target cassette optimized for maximal fluorescence intensity described in Chapter IV.

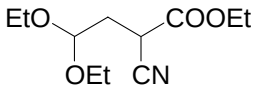
Isolation of the desired target compounds might be accomplished using HPLC. Once isolated, these rhodamine-based acceptors are potential components to be incorporated into through-bond energy transfer cassettes for intracellular imaging (Figure 5.2).

In summary, this thesis describes the synthesis of a nucleoside analogue, and donor and acceptor components to be incorporated into through-bond energy transfer systems designed for DNA sequencing and other applications in biotechnology.

CHAPTER VI

EXPERIMENTAL SECTION

General Procedures. High field NMR spectra were recorded on Inova 300 (^{1}H at 300 MHz, ^{13}C at 75 MHz) and Mercury 300 (^{1}H at 300 MHz) NMR spectrometers. Chemical shifts are reported in units of ppm relative to solvent ($CDCl_3$: 7.27 ppm for ^{1}H and 77.0 for ^{13}C; D_2O: 4.63 ppm for ^{1}H; DMSO-*d*$_6$: 2.50 ppm for ^{1}H and 39.5 for ^{13}C; CD_3OD: 3.30 ppm for ^{1}H and 49.0 for ^{13}C; Acetone-*d*$_6$: 2.04 ppm for ^{1}H and 29.9 for ^{13}C). Mass spectra were obtained from the Mass Spectrometry Applications Laboratory at Texas A&M University. Thin layer chromatography was performed using silica gel 60 F254. Flash chromatography was performed using silica gel (230-600 mesh). CH_2Cl_2, THF, DMF, MeOH, CH_3CN, Et_2O, pyridine, triethylamine, and toluene were distilled from appropriate drying agents. Other chemicals were purchased from commercial suppliers and used as received.



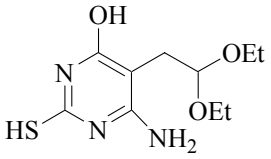
3

Diethoxyethylcyanoacetate (3). A mixture of bromoacetal **1** (320 g, 1.62 moles), ethylcyanoacetate **2** (912 g, 8.07 mol), anhydrous potassium carbonate (224 g), and sodium iodide (16 g) was stirred under reflux at 145–148 ^{o}C until the evolution of carbon dioxide ceased and then for a further 4 h at 145–148 ^{o}C. The mixture was cooled and dissolved in water (1600 mL) and Et_2O (1600 mL). The organic layer was washed with water, and the aqueous solution was extracted with Et_2O. The combined organic layers were dried over $MgSO_4$. Evaporation of the solvent under reduced pressure, and purification of the crude product by vacuum distillation gave the ester **3** (111 g, 30 %) as a colorless oil, bp 111-115 ^{o}C/1.3 mm Hg: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 1.20

(t, *J* = 7 Hz, 6H), 1.30 (t, *J* = 9Hz, 3H), 2.23 (t, *J* = 6.0 Hz, 2H), 3.50 (m, 1H), 3.65 (m, 4H), 4.23 (q, 2H), 4.70 (t, *J* = 6.0 Hz, 1H).

Literature Data: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 1.20 (t, 6H), 1.32 (t, 3H), 2.23 (t, 2H), 3.43 (m, 1H), 3.60 (m, 4H), 4.23 (q, 2H), 4.67 (t, 1H).

Seela, F.; Richter, R. *Chem. Ber.* **1978**, *111*, 2925-2930.

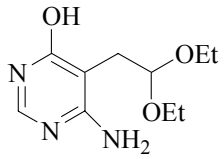


4

6-Amino-5-(2,2-diethoxy-ethyl)-2-mercapto-pyrimidin-4-ol (4). To a solution of sodium ethoxide (from 0.45 g of Na) in absolute ethanol (10 mL) was added thiourea (0.9 g, 11.8 mmol), followed by the ester **3** (2.29 g, 10 mmol). After the reaction mixture was heated at reflux for 12 h, it was cooled to room temperature and treated with glacial acetic acid (1.2 mL) and water (12.5 mL). The precipitated solid was collected by filtration to give the acetal **4** (2.00 g, 80 %) as a white solid: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.10 (t, *J* = 7.0 Hz, 6H), 2.43 (d, *J* = 6.2 Hz, 2H), 3.40-3.60 (m, 4H), 4.50 (t, *J* = 6.2 Hz, 1H), 6.05 (s, 2H), 11.4 (s, 1H), 11.7 (s, 1H).

Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.07 (t, *J* = 7 Hz, 6H), 2.43 (d, *J* = 6 Hz, 2H), 3.42-3.45 (m, *J* = 7 Hz , 4H), 4.48 (t, *J* = 6 Hz, 1H), 6.03 (s, 2H), 11.4 (s, 1H), 11.7 (s, 1H).

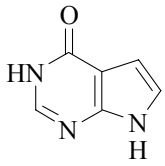
Seela, F.; Richter, R. Chem. Ber. **1978**, *111*, 2925-2930.



5

6-Amino-5-(2,2-diethoxy-ethyl)-pyrimidin-4-ol (5). To a solution of **4** (2.59 g, 10 mmol), water (125 mL), and ammonium hydroxide (8 mL) was added Raney nickel (8 mL). After the reaction mixture was heated at reflux for 5 h with stirring, it was filtered while hot and evaporated under reduced pressure to give the title compound **5** (2.00 g, 90 %) as a white solid: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.18 (t, J = 9.0 Hz, 6H), 2.65 (d, J = 6.3 Hz, 2H), 3.5-3.8 (m, 4H), 4.6 (t, J = 6.3 Hz, 1H), 6.10 (s, 2H), 7.75 (s, 1H).

Davoll, J. *J. Chem. Soc.* **1960**, 131-138.

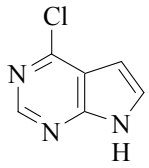


6

3,7-Dihydro-pyrrolo[2,3-*d*]pyrimidin-4-one (6). To a solution of compound **5** (1.0 g, 4.4 mmol) in water (10 mL) was added 0.2 mL of aqueous HCl. The reaction mixture was stirred at room temperature for 36 h. The solid compound was filtered and dried in the oven to give the pyrimidone **6** (380 mg, 65 %) as a white solid: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 6.45 (d, J = 2.4 Hz, 1H), 7.02 (d, J = 2.4 Hz, 1H), 7.85 (s, 1H).

Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 6.42 (m, 1H), 7.00 (m, 1H), 7.82 (s, 1H).

Seela, F.; Richter, R. *Chem. Ber.* **1978**, *111*, 2925-2930.

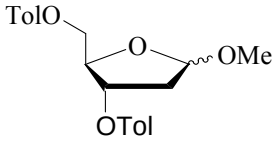


7

4-Chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine (7). A mixture of compound **6** (800 mg, 5.9 mmol) and phosphoryl chloride (8 mL) was refluxed for 4 h. Phosphoryl chloride was removed *in vacuo*, and the residue was treated with crushed ice, neutralized by $NaHCO_3$ to pH 7 and then extracted with ether (4 x 25 mL). The ether extracts were combined, dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to give the desired product **7** (360 mg, 40 %) after recrystallization from EtOAc as a white solid: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 6.61 (d, J = 3.6 Hz, 1H), 7.70 (d, J = 3.6 Hz, 1H), 8.60 (s, 1H); MS (ESI): m/z 154 $(M+H)^+$.

Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 6.60 (d, J = 4 Hz, 2Hz, 1H), 7.75 (d, J = 4 Hz, 2.5 Hz, 1H), 8.60 (s, 1H);

Lupke, U.; Seela, F. *Chem. Ber.* **1979**, *112*, 3526-3529.



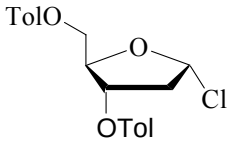
10

1*α*/*β*-*O*-Methyl-2-deoxy-D-ribofuranosyl bis(*p*-toluate) (10). To a solution of 2-deoxy-D-ribose **8** (5 g, 37.3 mmol) in MeOH (60 mL) was added 1 % methanolic HCl (10 mL prepared by adding 0.9 mL of acetyl chloride to 50 mL of MeOH). The reaction mixture was stirred at room temperature for 30 min and neutralized to pH 7 by sodium bicarbonate. After filtration, the methanol was removed by repeated coevaporation with pyridine (1 x 25 mL and 2 x 12 mL) to give the crude compound **9**, which was used without any purification for acylation. The residue was dissolved in pyridine (30 mL),

cooled to 0 ^{o}C and *p*-toluoyl chloride was added dropwise. The reaction mixture was stirred at room temperature overnight. The solution was then diluted with cold water (75 mL) and extracted 3 times with CH_2Cl_2. The organic extracts were combined, washed twice with $NaHCO_3$, once with 2N HCl, and once with water, then dried over anhydrous $NaHCO_3$ and evaporated under reduced pressure to furnish the crude mixture of bis(4-methyl benzoates) **10**. The α-isomer was isolated by flash chromatography eluting with CH_2Cl_2 to give the pure **10** (9.00 g, 63 %) as light yellow oil: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 2.15, 2.30 (m, 2H), 2.40 (s, 6H), 3.40 (s, 3H), 4.50 (m, 3H), 5.10 (dd, 1H), 5.30 (dt, 1H), 7.20, 7.90 (m, 8H).

Literature Data: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 2.20, 2.50 (m, 2H), 2.37 (s, 6H), 3.41 (s, 3H), 4.57 (bt, 3H), 5.15 (dd, 1H), 5.35 (dt, 1H), 7.15, 7.98 (m, 8H).

Takeshita, M; Chang C.; Johnson F.; Will, S.; Grollman, A. P. *J. Biol. Chem.* **1987**, *262*, 10171-10179.

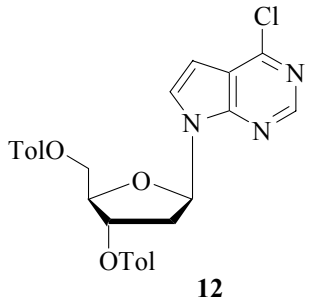


11

1*α*-Chloro-2-deoxy-D-ribofuranosyl bis(*p*-toluate) (11). To a solution of **10** (3.84 g, 9.9 mmol) in acetic acid (6 mL) was slowly added a saturated solution of HCl in acetic acid (9.6 mL prepared by adding 2.4 mL of acetyl chloride to a solution of 12.2 mL acetic acid and 0.6 mL water on cooling). After an additional amount of acetyl chloride (0.8 mL) was added, the chloride precipitated. The crystals were collected by filtration, washed with cold ether, and dried *in vacuo* to give the chloride **11** (2.2 g, 57 %) as a white crystalline solid: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 2.41 (s, 6H), 2.80 (m, 2H), 4.60 (m, 2H), 4.80 (m, 1H), 5.60 (dt, 1H), 6.50 (dd, J = 4.3 Hz, 0.6 Hz, 1H), 7.20 (d, J = 8.4 Hz, 4H) 7.90, 8.10 (dd, J = 8.4 Hz, 1.2 Hz, 4H).

Literature Data: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 2.41 (s, 6H), 2.75 (m, 2H), 4.60 (bd, 2H), 4.78 (m, 1H), 5.54 (dt, 1H), 6.45 (dd, J = 4.0 Hz, 0.5 Hz, 1H), 7.17, 8.04 (dd, 8H).

Takeshita, M; Chang C.; Johnson F.; Will, S.; Grollman, A. P. *J. Biol. Chem.* **1987**, *262*, 10171-10179.

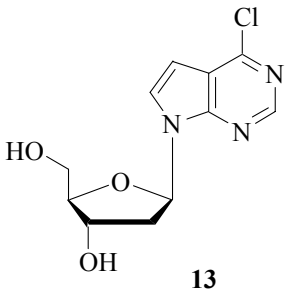


12

4-Chloro-7-(2-deoxy-3,5-di-*O*-(*p*-toluoyl)-*β*-D-*erythro*-pentofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (12). To a suspension of compound **7** (1.02 g, 6.65 mmol) in dry acetonitrile (42 mL) was added sodium hydride (50 % in oil, 0.28 mg) and the mixture was stirred at room temperature for 30 min. Compound **11** was added portionwise with stirring. The reaction mixture was further stirred at 50 ^{o}C for 8 h and then was filtered to remove any insoluble material. The filtrate was evaporated under reduced pressure and the oily residue was purified by flash chromatography eluting with 20 % EtOAc/hexanes. Crystallization from ethanol yielded the title compound **12** (2.4 g, 71 %) as white crystals: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 2.41, 2.49 (s, 6H), 2.78 (m, 1H), 3.18 (m, 1H), 4.56 (m, 3H), 5.76 (m, 1H), 6.75 (d, J = 3.5 Hz, 1H), 6.76 (m, 1H), 7.35, 7.90 (m, 9H), 8.65 (s, 1H), 8.65 (s, 1H); ^{13}C-NMR (DMSO-d_6, 75 MHz): δ (ppm) 21.9, 36.7, 64.8, 75.5, 82.1, 84.3, 100.8, 118.3, 129.4, 151.4, 151.6, 151.7, 166.5; MS (ESI): m/z 506 $(M+H)^+$.

Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.37, 2.40 (s, 6H), 2.77 (m, 1H), 3.18 (m, 1H), 4.60 (m, 3H), 5.77 (m, 1H), 6.75 (d, J = 3.7 Hz, 1H), 6.78 (m, 1H), 7.34, 7.91 (m, 9H), 8.65 (s, 1H), 8.65 (s, 1H); ^{13}C-NMR (DMSO-d_6, 75 MHz): δ (ppm) 21.3, 36.1, 64.2, 75.0, 81.5, 84.0, 100.2, 117.7, 128.7, 150.7, 151.0, 151.1, 166.5.

Seela, F.; Muth, H.-P.; Bindig, U. *Synthesis* **1988**, 670-674.

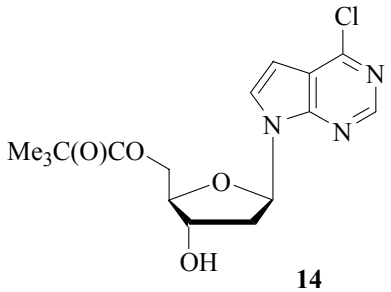


13

4-Chloro-7-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (13). Compound **12** (7.3 g, 14.3 mmol) was added to a solution of 7N NH_3/MeOH (300 mL). The reaction mixture was stirred at room temperature for 5 d. The solution was evaporated *in vacuo* and the residue was adsorbed into silica and purified by flash chromatography eluting with 10 % MeOH/CH_2Cl_2 to give the target compound **13** (2.7 g, 70 %) as a white solid: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.10, 2.30 (m, 2H), 3.55 (m, 2H), 3.85 (m, 1H), 4.38 (m, 1H), 4.97 (m, J = 5.0 Hz, 1H), 5.30 (m, J = 3.9 Hz, 1H), 6.58 (m, 1H), 6.60 (d, J = 3.9 Hz, 1H), 7.70 (d, J = 3.6 Hz, 1H), 8.44 (s, 1H); ^{13}C-NMR (DMSO-d_6, 75 MHz): δ (ppm) 39.9, 62.5, 71.8, 84.0, 88.0, 100.7, 118.0, 129.5, 152.0, 153.0, 153.5; MS (ESI): m/z 270 $(M+H)^+$.

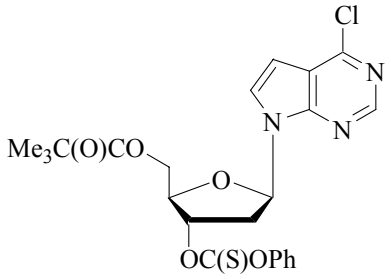
Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.18, 2.40 (m, 2H), 3.57 (m, 2H), 3.87 (m, 1H), 4.40 (m, 1H), 5.00 (m, J = 5.4 Hz, 1H), 5.35 (m, J = 4.2 Hz, 1H), 6.66 (m, 1H), 6.72 (d, J = 3.8 Hz , 1H), 7.99 (d, J = 3.88 Hz , 1H), 8.66 (s, 1H); ^{13}C-NMR (DMSO-d_6, 75 MHz): δ (ppm) 39.9, 61.8, 70.9, 83.4, 87.7, 99.7, 117.4, 128.5, 150.5, 150.6, 150.8.

Seela, F.; Muth, H.-P.; Bindig, U. *Synthesis* **1988**, 670-674.



14

4-Chloro-7-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-5′-*O*-pivaloyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (14). To a solution of the diol **13** (2.6 g, 9.7 mmol) in pyridine (100 mL) was added pivaloyl chloride (1.75 g, 14.6 mmol) and the reaction mixture was stirred at 0 ^{o}C for 5 h. The solution was then evaporated under reduced pressure, and the residue was adsorbed onto silica and purified by flash chromatography eluting with 50 % EtOAc/Hexanes to give the title compound **14** (1.7 g, 50 %) as white crystals: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 1.23 (s, 9H), 2.45-2.70 (m, 2H), 4.20 (m, 2H), 4.30 (m, 1H), 4.60 (m, 1H), 6.68 (d, J = 3.6 Hz, 1H), 6.70 (m, 1H), 7.44 (d, J = 3.6 Hz, 1H), 8.66 (s, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 27.5, 39.2, 40.6, 64.0, 72.0, 84.2, 84.4, 101.0, 118.2, 126.5, 151.0, 151.1, 152.4, 178.9; MS (ESI): m/z 354 $(M+H)^{+}$. Cocuzza, A. J. *Tetrahedron Lett.* **1988**, *29*, 4061-4064.

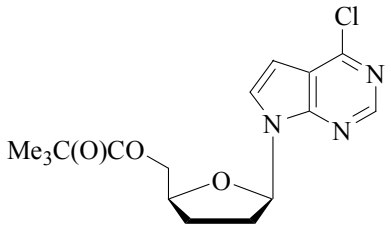


15

4-Chloro-7-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-5′-*O*-pivaloyl-3′-*O*-phenoxythiocarbonyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (15). To a solution of 5′-pivalate **14** (1.06 g, 3 mmol) in dichloromethane (21 mL), 4-dimethylaminopyridine (0.9 g, 7.5 mmol) and *O*-phenyl chlorothionocarbonate (0.8 mL, 5.9 mmol) were added. After

stirring the reaction mixture at room temperature for 4 h, additional dichloromethane (14 mL) was added, and the solution was washed with 0.5 N hydrochloric acid, 0.5 N sodium hydroxide, and brine. The combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure. The residue was adsorbed onto silica and purified by flash chromatography eluting with 40 % EtOAc/Hexanes to give the title compound **15** (1.3 g, 90 %) as white crystals: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 1.22 (s, 9H), 2.75-2.95 (m, 2H), 4.40 (m, 2H), 4.60 (m, 1H), 5.85 (m, 1H), 6.60 (d, *J* = 4.1 Hz, 1H), 6.80 (m, 1H), 7.10-7.30 (m, 5H), 7.40 (d, *J* = 4.1 Hz, 1H), 8.50 (s, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 27.5, 37.8, 39.1, 64.3, 81.8, 83.6, 84.7, 101.3, 118.6, 126.1, 151.2, 151.4, 152.7, 178.4, 194.3; MS (ESI): m/z 490 $(M+H)^+$.

Cocuzza, A. J. *Tetrahedron Lett.* **1988**, *29*, 4061-4064.

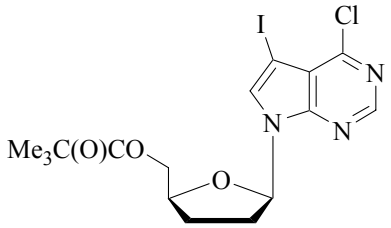


16

4-Chloro-7-(2,3-deoxy-*β*-D-*erythro*-pentofuranosyl)-5′-*O*-pivaloyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (15). To a stirred solution of **15** (1.7 g, 3.4 mmol) and 2,2′-azobis(2-methyl)propionitrile (AIBN) (0.1 g, 0.62 mmol) in 103 mL of dry toluene, was added tri-*n*-butyltin hydride (1.34 g, 4.6 mmol) and the reaction mixture was stirred at 110 ^{o}C for 1 h. After the reaction mixture had been cooled, it was diluted with 80 mL of ether and washed with 80 mL of 10 % aqueous potassium fluoride and brine. The combined organic layers were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was adsorbed onto silica and purified by flash chromatography eluting with 1.5 % MeOH/CH_2Cl_2 to give the title compound **16** (0.75 g, 64 %) as light yellow oil: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 1.20 (s, 9H), 2.10-2.50 (m, 4H), 4.25 (m, 2H), 4.40 (m, 1H), 6.53 (dd, *J* = 4.5 Hz, 2.4 Hz, 1H), 6.62 (d, *J* =

3.6 Hz, 1H), 7.51 (d, *J* = 3.6 Hz, 1H), 8.61 (s, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 26.8, 27.4, 32.6, 39.0, 65.5, 78.6, 85.2, 100.3, 118.3, 126.4, 150.8, 150.8, 152.2, 178.4; MS (ESI): m/z 338 $(M+H)^+$.

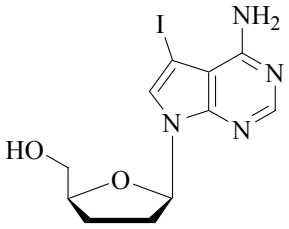
Cocuzza, A. J. *Tetrahedron Lett.* **1988**, *29*, 4061-4064.



17

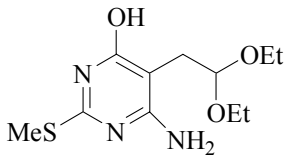
4-Chloro-5-iodo-7-(2,3-deoxy-*β*-D-*erythro*-pentofuranosyl)-5′-*O*-pivaloyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (15). To a solution of pivalate **16** (0.75 g, 2.2 mmol in 66 mL CH_2Cl_2) was added a solution of iodine monochloride (0.34 mL in 3.5 mL CH_2Cl_2). The reaction mixture was stirred at room temperature for 4 h and then was partitioned between dichloromethane and aqueous sodium hydrosulfite. The organic layer was washed with aqueous sodium bicarbonate and brine, dried over sodium sulfate, and evaporated under reduced pressure. The residue was triturated with CH_2Cl_2/ether to give the title compound **17** (935 mg, 90 %) as a light yellow solid: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 1.26 (s, 9H), 2.10-2.60 (m, 4H), 4.33 (m, 2H), 4.40 (m, 1H), 6.54 (dd, *J* = 3.6 Hz, 3.3 Hz, 1H), 7.68 (s, 1H), 8.63 (s, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 26.4, 27.5, 33.1, 39.2, 52.1, 65.1, 79.1, 85.5, 123.7, 131.8, 151.0, 151.5, 152.9; 178.6; MS (ESI): m/z 464 $(M+H)^+$.

Cocuzza, A. J. *Tetrahedron Lett.* **1988**, *29*, 4061-4064.



18

7-Iodo-2′,3′-dideoxy-7-deazaadenosine (18). Compound **17** (0.89 g, 1.9 mmol) was added to a solution of 7N NH_3/MeOH (160 mL) in a pressure flask well sealed. The reaction mixture was stirred at 120 ^oC for 18 h. The solution was evaporated *in vacuo* and the residue was adsorbed into silica and purified by flash chromatography eluting with 5% MeOH/CH_2Cl_2 to give the target compound **18** (630 mg, 90 %) as a white solid: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.10 - 2.40 (m, 4H), 3.50 (m, 2H), 4.10 (m, 1H), 4.95 (t, J = 5.4 Hz, 1H), 6.32 (dd, J = 6.6 Hz, 4.2 Hz, 1H), 6.65 (s, 2H), 7.67 (s, 1H); 8.09 (s, 1H) ^{13}C-NMR (DMSO-d_6, 75 MHz): δ (ppm) 26.5, 32.5, 52.0, 63.8, 81.7, 84.3, 103.7, 127.3, 150.1, 152.6, 157.8; MS (ESI): m/z 361 $(M+H)^+$.
Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.00 - 2.30 (m, 4H), 3.49, 3.59 (m, 2H), 4.04 (m, J = 3.5 Hz, 1H), 4.95 (t, J = 5.5 Hz, 1H), 6.34 (dd, J = 4.4 Hz, 6.8 Hz, 1H), 6.65 (s, 2H), 7.67 (s, 1H); 8.09 (s, 1H).
Hobbs, F. W.; Cocuzza, A. US Patent 5047519, 1988.



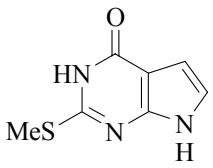
19

6-Amino-5-(2,2-diethoxy-ethyl)-2-methylsulfanyl-pyrimidin-4-ol (19).
Compound **4** (30 g, 0.132 mol) was dissolved in 1N NaOH (17 mL, 0.12 mol) and dimethyl sulfate (12.47 mL, 0.132 mol) was slowly added over a period of 10 min. The

reaction mixture was stirred at room temperature for 2 h. The precipitated solid was collected by filtration and washed with water to give the 2-methylthiopyrimidine **19** (25.5 g, 80 %) as a white solid: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 0.98 (t, J = 7.2 Hz, 6H), 1.98 (s, 3H), 2.4 (m, 2H), 3.2-3.5 (m, 4H), 4.45 (t, J = 6 Hz, 1H), 5.9 (s, 2H, NH_2), 11.6 (s, 1H, NH).

Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 1.10 (t, J = 7 Hz, 6H), 2.40 (m, 2H), 2.43 (s, 3H), 3.47 (m, 4H), 4.55 (t, J = 6 Hz, 1H), 6.07 (NH_2).

Seela, F.; Richter, R. *Chem. Ber.* **1978**, *111*, 2925-2930.

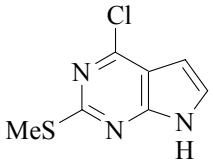


20

2-Methylsulfanyl-3,7-dihydro-pyrrolo[2,3-*d*]pyrimidin-4-one (20). In the same manner as for **6**, the title compound was prepared by using the compound **19** (2.73 g, 10 mmol), water (50 mL), aqueous HCl (1 mL). The solid compound was filtered and dried in the oven to give the title compound **20** (1.26 g, 70 %) as a white solid: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.50 (s, 3H), 6.30 (d, J = 3.8 Hz, 1H), 6.85 (d, J = 3.8 Hz, 1H).

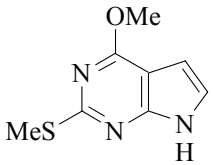
Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.53 (s, 3H), 6.37 (d, J = 4 Hz, 1H), 6.88 (d, J = 4 Hz, 1H).

Seela, F.; Richter, R. *Chem. Ber.* **1978**, *111*, 2925-2930.



21

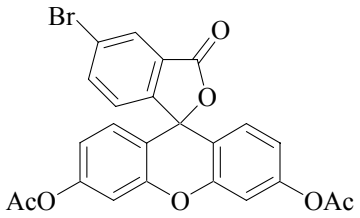
4-Chloro-2-methylsulfanyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (21). In the same manner as for **7**, the title compound was prepared by using the compound **20** (1.81 g, 1 mmol) and phosphoryl chloride (20 mL). The crude product was recrystallized from EtOAc to give the title compound **21** (0.76 g, 40 %) as a white solid: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.57 (s, 3H), 6.50 (d, J = 3.0 Hz, 1H), 7.56 (d, J = 3.0 Hz, 1H).
Literature data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.60 (s, 3H), 6.50 (m, 1H), 7.50 (m, 1H).
Lupke, U.; Seela, F. *Chem. Ber.* **1979**, *112*, 3432-3440.



22

4-Methoxy-2-methylsulfanyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (22). Compound **22** (1.99 g, 10 mmol) was suspended in dry MeOH (50 mL) containing sodium methoxide (1.24 g, 2.23 mmol), then the mixture was refluxed for 90 h. The reaction mixture was neutralized to pH 7 by Dowex basic resin. The resin was removed by filtration, and the filtrate was evaporated *in vacuo*. The crude product was recrystallized from 10 % aqueous MeOH to give the title compound **22** (1.36 g, 70 %) as fine colorless crystals: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 2.60 (s, 3H), 4.20 (s, 3H), 6.45 (d, J = 3.3 Hz, 1H), 7.0 (d, J = 3.3 Hz, 1H), 11.8 (s, 1H, -NH).
Literature data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.56 (s, 3H), 4.03 (s, 3H), 6.39 (d, J = 3.5 Hz, 1H), 7.18 (d, J = 3.5 Hz, 1H), 11.8 (s, -NH).

Lupke, U.; Seela, F. *Chem. Ber.* **1979**, *112*, 3432-3440.

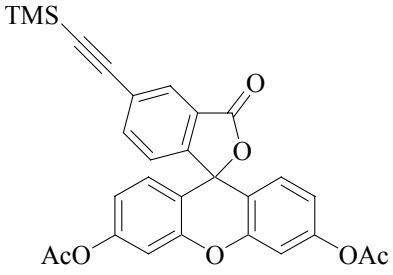


25

5-Bromofluorescein diacetate (26). A mixture of 4-bromophthalic anhydride **24** (20 g, 88 mmol) and resorcinol **23** (19.4 g, 176 mmol) was heated at 200 ^{o}C with stirring for 12 h. The reaction mixture was then cooled to room temperature, and the solid product was collected and ground in a mortar to afford 34 g of a mixture of 5- and 6-bromofluorescein. The crude bromofluorescein mixture, which was used further without any purification in the acylation, was dissolved in 140 mL of acetic anhydride and refluxed for 3 h. The reaction mixture was then cooled to room temperature and pale yellow crystals precipitated. The crystals were collected by filtration, rinsed with cold acetic anhydride and ethanol and dried *in vacuo* to give 11 g of a mixture of 5- and 6-isomers **25a** and **25b**, but with the 5-bromo isomer **25a** representing the major product. The mother liquor was concentrated to half the volume, allowed to stand for about 20 h, and the crystals formed were collected by filtration and dried in *vacuo*. The first and second crops of crystals were recrystallized from acetic anhydride twice to give regioisomerically pure 5-bromofluorescein diacetate **26** (12.2 g, 28 %) as a light brown solid: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 2.45 (s, 6H), 6.87 (m, 4H), 7.13 (dd, J = 1.5 Hz, 0.8 Hz, 2H), 7.83 (dd, J = 8.3 Hz, 1.5 Hz,1H), 8.19 (dd, J = 1.5 Hz, 0.8 Hz, 1H). Literature Data: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 2.33 (s, 6H), 6.84 (m, 4H), 7.09 (dd, J = 1.8 Hz, 0.6 Hz, 1H), 7.81 (dd, J = 8.1 Hz, 1.8 Hz, 1H), 8.17 (dd, J = 1.8 Hz, 0.6 Hz, 1H).

Jiao, G.-S.; Han, J. W.; Burgess, K. *J. Org. Chem.* **2003**, *68*, 8264-8267.

6-Bromofluorescein diacetate was also isolated. The mother liquors from the recrystallization of 5-bromofluorescein diacetate were combined and acetic anhydride was evaporated under reduced pressure. The solid was then dissolved in ethanol and allowed to stand overnight. The crystals formed were collected by filtration and recrystallized from ethanol to afford 6-bromofluorescein diacetate.

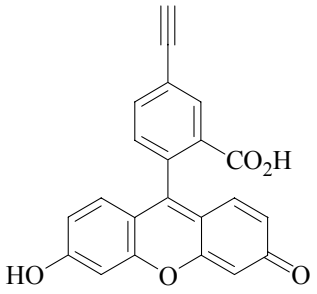


27

5-(2-Trimethylsilylethynyl)fluorescein diacetate (27). 5-Bromofluorescein diacetate **26** (500 mg, 1.0 mmol), copper iodide (9.5 mg, 0.05 mmol), and palladium tetrakis triphenylphosphine (35 mg, 0.05 mmol) were combined in 1 mL of THF in a Schlenk flask. Triethyl amine (1.4 mL, 10 mmol) was added to the above mixture, followed by trimethylsilyl acetylene (0.28 mL, 2 mmol) and the reaction mixture was stirred at 70 ^{o}C for 5h. The mixture was then cooled to room temperature and stirring was continued overnight. The solution was then filtered over Celite and washed with THF. The solvent was evaporated under reduced pressure, Et_2O was added and extracted with 0.1 M HCl (3 x 40 mL). The aqueous layers were extracted once with 25 mL of Et_2O, the organic extracts were combined and dried over anhydrous $MgSO_4$, and the solvent was evaporated under reduced pressure. The product was recrystallized from ethanol to afford 5-(2-trimethylsilylethynyl) fluorescein diacetate **27** (388 mg, 75 %) as a light brown powder. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 0.28 (s, 9 H), 2.32 (s, 6H), 6.82 (m, 4H), 7.10 (m, 2H), 7.14 (d, J = 8.3 Hz, 1H), 7.74 (dd, J = 8.3 Hz, 1.5 Hz, 1H), 8.10 (s, 1H).

Literature Data: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 0.29 (s, 9 H), 2.33 (s, 6H), 6.82 (m, 4H), 7.10 (m, 2H), 7.13 (d, *J* = 8.1 Hz, 1H), 7.75 (dd, *J* = 8.1 Hz, 1.2 Hz, 1H), 8.10 (s, 1H).

Jiao, G.-S.; Thoresen, L. H.; Burgess, K. *J. Am. Chem. Soc.* **2003**, *125*, 14668-14669.

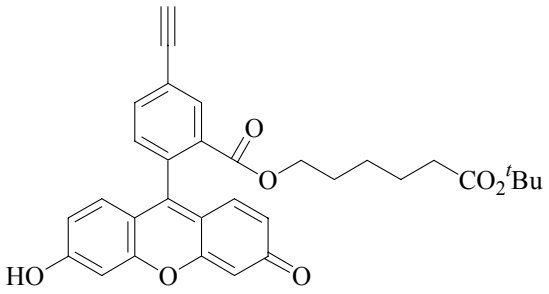


28

5-Ethynylfluorescein (28). 5-(2-Trimethylsilylethynyl)fluorescein diacetate **27** (318 mg, 0.62 mmol) and potassium carbonate (428 mg, 3.10 mmol) were combined in 6 mL of 1:1 MeOH/THF. After the reaction mixture was stirred for 20 h at 25 ^{o}C, it was poured into 40 mL of water and acidified with concentrated HCl to pH 2. The precipitate that formed upon acidification was collected by filtration, rinsed with water and dried *in vacuo* to afford the desired product **28** (177 mg, 80 %) as an orange solid. ^{1}H-NMR (acetone-d_6, 300 MHz): δ (ppm) 3.94 (s, 1H), 6.69 (dd, *J* = 8.3 Hz, 2.7 Hz, 2H), 6.75 (d, *J* = 8.3 Hz, 2H), 6.80 (d, *J* = 2.7 Hz, 2H), 7.35 (dd, *J* = 7.8 Hz, 0.8 Hz, 1H), 7.91 (dd, *J* = 7.8 Hz, 1.3 H, 1H), 8.06 (dd, *J* = 1.3 Hz, 0.4 Hz, 1H).

Literature Data: ^{1}H-NMR (acetone-d_6, 500 MHz): δ (ppm) 3.91 (s, 1H), 6.62 (dd, *J* = 8.5 Hz, 2.5 Hz, 2H), 6.70 (d, *J* = 8.5 Hz, 2H), 6.75 (d, *J* = 2.5 Hz, 2H), 7.31 (dd, *J* = 8 Hz, 1 Hz, 1H), 7.87 (dd, *J* = 8 Hz, 1.5 Hz, 1H), 8.01 (dd, *J* = 1.5 Hz, 0.5 Hz, 1H).

Jiao, G.-S.; Thoresen, L. H.; Burgess, K. *J. Am. Chem. Soc.* **2003**, *125*, 14668-14669.

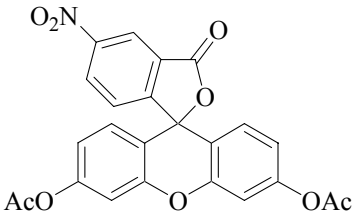


29

5-Ethynylfluorescein-(5-*tert*-butoxycarbonyl)-pentyl ester (29). To a solution of 5-ethynylfluorescein **28** (168 mg, 0.47 mmol) and *tert*-butyl ester of 6-bromohexanonoic acid (148 mg, 0.58 mmol) in 4.5 mL of DMF was added potassium carbonate (85 mg, 0.61 mmol). After the reaction mixture was stirred at 25 ^{o}C for 24 h, it was adsorbed onto silica, then purified by flash chromatography using 10 % MeOH/$CHCl_3$ to give **29** (151 mg, 60 %) as an orange solid: ^{1}H-NMR (CD_3OD, 300 MHz): δ (ppm) 0.9 – 1.06 (m, 2H), 1.24 – 1.30 (m, 2H), 1.37 – 1.40 (m, 2H), 1.44 (s, 9H), 2.10 (t, *J* = 7.5 Hz, 2H), 3.84 (s, 1H), 3.95 (t, *J* = 6 Hz, 2H), 6.69 (dd, *J* = 9 Hz, 1.8 Hz, 2H), 6.73 (d, *J* = 2.0 Hz, 2H), 7.02 (d, *J* = 9 Hz, 2H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.89 (dd, *J* = 7.8 Hz, 1.2 Hz, 1H), 8.30 (d, *J* = 1.2 Hz, 1H); ^{13}C-NMR (CD_3OD, 75 MHz): δ (ppm) 24.59, 25.30, 27.37, 27.95, 34.98, 65.71, 78.30, 80.30, 80.81, 81.40, 103.41, 115.20, 121.80, 124.80, 130.73, 130.97, 131.25, 134.00, 134.43, 135.78, 154.25, 157.75, 165.00, 173.60.

Literature Data: ^{1}H-NMR (CD_3OD, 500 MHz): δ (ppm) 0.9 - 1.01 (m, 2H), 1.23 – 1.29 (m, 2H), 1.35 – 1.41 (m, 2H), 1.42 (s, 9H), 2.08 (t, *J* = 7.3 Hz, 2H), 3.83 (s, 1H), 3.94 (t, *J* = 7.3 Hz, 2H), 6.70 (dd, *J* = 9.3 Hz, 2.0 Hz, 2H), 6.73 (d, *J* = 2.0 Hz, 2H), 7.01 (d, *J* = 9.3 Hz, 2H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.88 (dd, *J* = 7.8 Hz, 1.5 Hz, 1H), 8.30 (d, *J* = 1.5 Hz, 1H); ^{13}C-NMR (CD_3OD, 125 MHz): δ (ppm) 25.7, 26.4, 28.4, 29.1, 36.0, 66.8, 81.3, 81.4, 81.9, 82.5, 104.4, 116.3, 122.8, 125.9, 131.9, 132.1, 132.4, 135.1, 135.5, 136.9, 155.5, 158.9, 166.1, 174.7.

Jiao, G.-S.; Thoresen, L. H.; Burgess, K. *J. Am. Chem. Soc.* **2003**, *125*, 14668-14669.

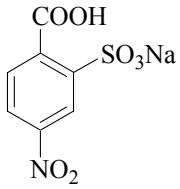


32

5-Nitrofluorescein diacetate (32). 4-Nitrophthalic anhydride **30** (5 g, 25.5 mmol) and 1,3-dihydroxybenzene **23** (5.6 g, 51 mmol) were dissolved in 75 mL of methanesulfonic acid. After the reaction mixture was stirred at 85 ^{o}C for 72 h, the mixture was cooled to room temperature and then added in small portions to 425 mL of an ice water mixture. The precipitate was collected by filtration and dried in the oven to give a mixture of 5- and 6-nitrofluorescein **31a** and **31b**. The crude nitrofluorescein mixture was used further without any purification in the acylation reaction. In the same manner as for **26**, the 5-nitrofluorescein diacetate was isolated by repeated recrystallization from acetic anhydride to afford pure **32** (3.5 g, 30 %) as a light brown powder. ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.33 (s, 6H), 6.98-7.10 (m, 4H), 7.36 (d, J = 2.1 Hz, 2H), 7.80 (d, J = 9 Hz, 1H), 8.64 (dd, J = 6.3 Hz, 4.2 Hz, 1H), 8.76 (d, J = 9 Hz, 1H).

Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 2.28 (s, 6H), 6.52 – 7.05 (m, 4H), 7.30 (d, 2H), 7.75 (d, 1H), 8.55 (dd, 1H), 8.70 (d, 1H).

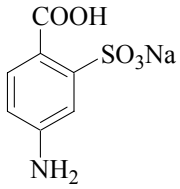
Sigmund, H; Pfleiderer, W. *Helv. Chim. Acta* **2003**, *86*, 2299-2334.



34

Sodium 5-nitro-2-carboxy-benzenesulfonate (34). A mixture of 2-methyl-5-nitrobenzenesulfonic acid **33** (25.0 g, 115 mmol) and NaOH (3.95 g, 98.9 mmol) was dissolved in 170 mL of water with heating. Potassium permanganate (70.4 g, 445.43 mmol in 250 mL of water) was added, and the reaction mixture was stirred with heating for 3 h. The brown suspension was filtered through Celite and the filtrate was concentrated *in vacuo* to 100 mL. The mixture was cooled to room temperature, and the crystals were collected by filtration and rinsed with H_2O, EtOH, and then Et_2O. The mother liquor was concentrated to 50 mL and cooled to room temperature, then the crystals were collected by filtration. The first and the second crops were combined and dissolved in 100 mL of water and 8 mL of concentrated HCl with warming. Then 150 mL of EtOH was added to the hot solution and it was then allowed to stand at room temperature overnight. The crystals were collected by filtration and washed with cold 50/50 EtOH/H_2O, EtOH, and then Et_2O to give **34** (20.4 g, 66 %) as white needles: ^{1}H-NMR (D_2O, 300 MHz): δ (ppm) 7.36 (d, J = 8.5 Hz, 1H), 8.18 (dd, J = 3.6 Hz, 2.5 Hz, 1H), 8.46 (d, J = 2.5 Hz, 1H); ^{13}C-NMR (D_2O, 75 MHz): δ (ppm) 122.86, 126.33, 128.12, 139.29, 144.59, 146.52, 175.11.

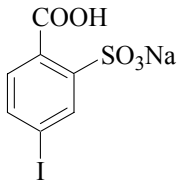
Conrow, R. B.; Bernstein, S., US Patent 4608205, 1986.



35

Sodium 5-nitro-2-carboxy-benzenesulfonate (35). Sodium 5-nitro-2-carboxy-benzenesulfonate **34** (0.5 g, 1.85 mmol) was dissolved in H_2O (5 mL) and Pd/C (10 %, 0.19 g, 0.185 mmol) was then added. The suspension was flushed with H_2 and stirred at 80 ^oC for 3 h. The black suspension was then filtered through Celite and the filtrate was concentrated *in vacuo* to give **35** (0.35 g, 80 %) as a white solid: ^{1}H-NMR (D_2O, 300 MHz): δ (ppm) 6.75 (dd, J = 5.5 Hz, 2.5 Hz, 1H), 7.00 (d, J = 8.5 Hz, 1H), 7.02 (d, J = 2.5 Hz, 1H); ^{13}C-NMR (D_2O, 75 MHz): δ (ppm) 114.08, 118.73, 128.41, 130.28, 139.10, 146.29, 177.88.

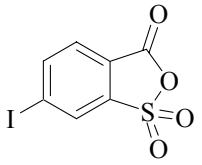
Conrow, R. B.; Bernstein, S., US Patent 4608205, 1986.



36

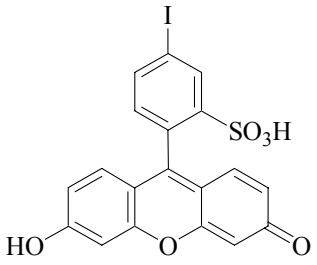
Sodium 2-carboxy-5-iodo-benzenesulfonate (36). To a solution of sodium 5-nitro-2-carboxy-benzenesulfonate **35** (0.50 g, 2.09 mmol) in 7 mL of 12 N HCl was added 3.6 g ice and the reaction mixture was cooled and stirred in an ice/water bath. Sodium nitrite (0.18 g, 2.6 mmol) in 3.5 mL was added dropwise over 2 min and the mixture was stirred at 0 ^oC for 30 min. Potassium iodide (3.45 g, 20.8 mmol) in 6 mL of water was added dropwise over 2 min with vigorous stirring at 0 ^oC. The cooling bath was removed and the reaction mixture was stirred for a further 1 h at room temperature. The mixture was extracted three times with 25 % *i*PrOH/$CHCl_3$, then the combined

organic extracts were washed with saturated aqueous $Na_2S_2O_3$ and dried over anhydrous Na_2SO_4. The filtrate was evaporated *in vacuo* to afford the title compound **36** (0.47 g, 65 %) as an off-white solid: ^{1}H-NMR (D_2O, 300 MHz): δ (ppm) 6.95 (dd, *J* = 2.6 Hz, 5.7 Hz, 1H), 7.45 (d, *J* = 2.6 Hz, 1H), 7.82 (d, *J* = 8.5 Hz, 1H).
Conrow, R. B.; Bernstein, S., US Patent 4608205, 1986.



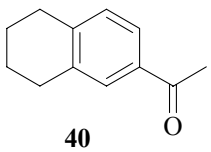
37

5-Iodo-2-sulfobenzoic anhydride (37). A mixture of sodium 5-iodo-2-sulfobenzoate **36** (1 g, 2.85 mmol) and thionyl chloride (2.1 mL, 28.57 mmol) was heated at reflux for 7 h. The reaction mixture was then cooled to room temperature and thionyl chloride was distilled off. To the remaining mixture was added 10 mL of benzene and the solution was boiled for 1 h. The mixture was filtered while hot and the solid was washed with hot benzene (2 x 5 mL). The filtrates were combined and the solution was concentrated *in vacuo* until 2 mL of solution remained. The solution was then cooled in an ice bath, the crystals were collected by filtration, and the crude product was recrystallized from benzene to give **37** (0.26 g, 30 %) as an off-white solid: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 7.84 (dd, *J* = 8.1, 0.6 Hz, 1H), 8.29 (dd, *J* = 8.1 Hz, 1.5 Hz, 1H), 8.36 (dd, *J* = 0.6 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 105.2, 124.3, 128.4, 131.3, 141.2, 144.4, 154.00; MS (ESI): m/z 311 $(M+H)^+$.
Literature Data: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 7.84 (dd, *J* = 8.1 Hz, 0.5 Hz, 1H), 8.30 (dd, *J* = 8.1 Hz, 1.2 Hz, 1H), 8.36 (dd, *J* = 1.2 Hz, 0.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 105.33, 124.70, 128.61, 131.44, 141.34, 144.77, 154.91; MS (ESI): m/z 311 $(M+H)^+$.
Jiao, G.-S.; Han, J. W.; Burgess, K. *J. Org. Chem.* **2003**, *68*, 8264-8267.



38

5-Iodosulfofluorescein (38). 5-Iodo-2-sulfobenzoic anhydride **37** (27.7 mg, 0.09 mmol) and 1,3-dihydroxybenzene **23** (19.6 mg, 0.18 mmol) were dissolved in 0.2 mL of methanesulfonic acid. After the reaction mixture was stirred at 85 ^{o}C for 22 h, it was cooled to room temperature and then was poured into a minimum amount of water. The precipitated solid was collected by filtration, then dissolved in 10 % NaOH solution, and filtered again. The filtrate was acidified with concentrated HCl. A precipitate formed upon acidification and was collected by filtration, and dried *in vacuo* to afford **38** (38 mg, 90 %) as a reddish solid: ^{1}H-NMR (acetone-d_6, 300 MHz): δ (ppm) 6.68 (d, J = 7.8 Hz, 1H), 6.74 (dd, J = 8.8 Hz, 2.5 Hz, 2H), 6.79 (d, J = 2.5 Hz, 2H), 7.36 (d, J = 8.8 Hz, 2H), 7.90 (dd, J = 7.8 Hz, 1.3 Hz, 1H), 8.10 (d, J = 1.3 Hz 1H).
Literature Data: ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) 7.11 (d, J = 7.9 Hz, 1H), 7.18 (dd, J = 9.2 Hz, 2.2 Hz, 2H), 7.33 (d, J = 2.2 Hz, 2H), 7.44 (d, J = 9.2 Hz, 2H), 7.98 (dd, J = 7.9 Hz, 1.8 Hz, 1H), 8.21 (d, J = 1.8 Hz, 1H).
Jiao, G.-S.; Han, J. W.; Burgess, K. *J. Org. Chem.* **2003**, *68*, 8264-8267.



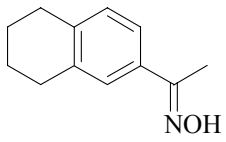
40

Acetyltetralin (40). To a cooled solution of tetralin **39** (5 g, 37.82 mmol) and acetyl chloride (3.03 g, 38.6 mmol) in 23 mL of benzene was added anhydrous aluminum chloride (5.7 g, 42.7 mmol) in small portions over a period of 10 min, while

the temperature of the mixture was held below 10 ^{o}C. The reaction mixture was stirred until the hydrogen chloride evolution ceased. The mixture was then poured into ice, and acetyltetralin **40** (4.8 g, 80 %) was isolated by vacuum distillation as light yellow oil: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 1.82 (m, 4H), 2.58 (s, 3H), 2.81 (m, 4H), 7.15 (d, *J* = 8.1 Hz, 1H), 7.67 - 7.70 (dd, *J* = 8.1 Hz, 2.7 Hz, 2H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 23.03, 23.23, 26.67, 29.59, 29.84, 125.63, 129.41, 129.51, 134.90, 137.52, 143.28, 198.14.

Literature Data: ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 1.79 – 1.84 (m, 4H), 2.57 (s, 3H), 2.78 – 2.84 (m, 4H), 7.14 (d, *J* = 8.4 Hz, 1H), 7.64 – 7.69 (m, 2H); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ (ppm) 22.94, 23.08, 26.57, 29.47, 29.73, 125.27, 129.06, 129.15, 134.54, 137.24, 143.00, 197.88.

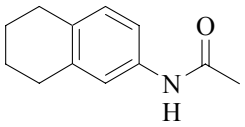
Kido, Y.; Yonehara, F.; Yamaguchi, H. *Tetrahedron* **2001**, *57*, 827-833.



41

6-Acetyltetralin oxime (41). To a solution of **40** (9.44 g, 54.18 mmol) in ethanol (50 mL) was added pyridine (13 mL, 162.5 mmol) and hydroxylamine hydrochloride (11.3 g, 162.5 mmol). The reaction mixture was stirred at 60 ^{o}C for 1 h and then the solution was concentrated *in vacuo*. The residue was then diluted with Et_2O, washed with water (3 x mL), dried over anhydrous $MgSO_4$, and the solvent was evaporated under reduced pressure. The solid was further recrystallized from ethyl acetate to afford g of **41** (9.7 g, 95 %) as white crystals: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 1.82 (m, 4H), 2.27 (s, 3H), 2.80 (m, 4H), 7.08 (d, *J* = 7.2 Hz, 1H), 7.33 (d, 7.2 Hz, 1H), 7.38 (s, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 12.60, 23.36, 23.39, 29.52, 29.75, 123.42, 127.04, 129.55, 134.02, 137.52, 138.87, 156.37; MS (ESI): m/z 190 $(M+H)^{+}$.

Brown, A. T.; Hallas G.; Lawson, R. *Chem. Ind.* **1981**, 248-249.

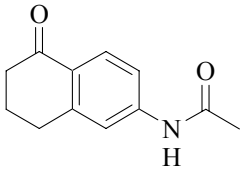


42

6-Acetamidotetralin (42). To a solution of **41** (5 g, 26.4 mmol) in 50 mL of pyridine was slowly added 6 mL of benzenesulphonyl chloride. The reaction mixture was stirred for 3 hours and the temperature was kept below 15 ^{o}C. The solution was then poured into a mixture of ice and 1M hydrochloric acid (~ 100 mL). After several hours, 6-acetamidotetralin separated, was washed with water, and filtered to yield of 6-acetamidotetralin **42** (3.0 g, 60 %) as a white solid: ^{1}H-NMR (acetone-d_6), 300 MHz): δ (ppm) 1.80 (m, 4H), 2.08 (s, 3H), 2.72 (m, 4H), 7.00 (d, J = 8.2 Hz, 1H), 7.34 (d, J = 8.2 Hz, 1H), 7.38 (s, 1H), 9.00 (1H, s, NH); ^{13}C-NMR (DMSO-d_6, 75 MHz): δ (ppm) 23.46, 23.58, 24.64, 28.93, 29.70, 117.42, 119.93, 129.60, 131.82, 137.20, 137.49, 168.7; MS (ESI): m/z 190 $(M+H)^{+}$.

Literature Data: ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.66 – 2.00 (m, 4H), 2.16 (s, 3H), 2.60 – 2.90 (m, 4H), 7.00 (d, J = 8 Hz, 1H), 7.23 (d, J = 8 Hz, 1H), 7.30 (s, 1H), 8.00 (1H, s, NH).

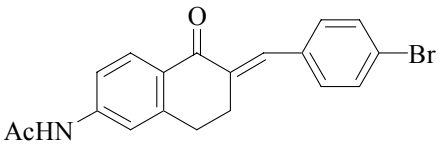
Daly, C. M..; Iddon, B; Suschitzky, H. *J. Chem. Soc. Perkin Trans I* **1988**, *7*, 1933-1938.



43

6-Acetamidotetralone (43). To a solution of 6-acetamidotetralin **42** (2 g, 10.5 mmol) in 4.8 mL of acetic acid and 1.4 mL of acetic anhydride was added a solution of chromium trioxide (1.38 g, 13.8 mmol) in 1 mL of water and 4 mL of acetic acid, while the temperature of the reaction mixture was kept below 10 ^{o}C by external cooling. After the solution was stirred overnight, it was poured into 70 mL of ice-water mixture and filtered. The crude product was adsorbed onto silica, then purified by flash chromatography eluting with 40% EtOAc/Hexanes to afford **43** (0.9 g, 40 %) as a pale yellow solid: ^{1}H-NMR (CD_3OD, 300 MHz): δ (ppm) 2.12 (m, 2H), 2.17 (s, 3H), 2.64 (t, *J* = 6.0 Hz, 2H), 2.95 (t, *J* = 6.3 Hz, 2H), 7.48 (d, *J* = 8.7 Hz, 1H), 7.66 (s, 1H), 7.94 (d, *J* = 8.7 Hz, 1H); ^{13}C-NMR (acetone-d_6, 75 MHz): δ (ppm) 23.50, 23.80, 28.50, 38.80, 117.20, 118.00, 128.10, 128.20, 144.10, 142.5, 168.80, 196.00; MS (ESI): m/z 204 $(M+H)^{+}$.

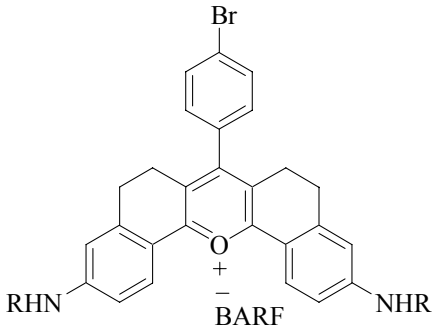
Allinger, N. L.; Jones, E. S. *J. Org. Chem.* **1962**, *27*, 70-76.



45

6-Acetamido-2-benzylidene-1-tetralone (45). 6-Acetamidotetralone **43** (0.5 mg, 2.45 mmol) and 4-bromobenzaldehyde (0.45 g, 2.45 mmol) were treated with 3 mL of 4 % ethanolic potassium hydroxide at 25 ^{o}C. The crystals were deposited almost immediately. After 2 hours, the solution was neutralized with acetic acid, water was

added, and the crystals were filtered. The crude product was then recrystallized from ethanol to give **45** (0.7 g, 85 %) as orange crystals: ^{1}H-NMR (acetone-d_6, 300 MHz): δ (ppm) 2.09 (s, 3H), 2.98 (t, J = 6.3 Hz, 2H), 3.12 (t, J = 6.3 Hz, 2H), 7.50 - 7.70 (m,7H), 7.81 (s, 1H), 8.05 (dd, J = 6 Hz, 2.6 Hz, 1H);
^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 25.13, 27.39, 29.29, 117.97, 118.24, 122.96, 129.70, 129.98, 131.61 (2 CH), 131.94 (2 CH), 134.96, 135.41, 136.25, 142.61, 145.29, 168.90, 186.90; MS (ESI): m/z 370/372 $(M+H)^+$.

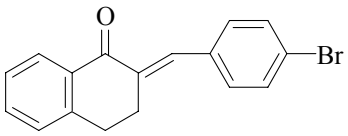


R = H, Ac

46

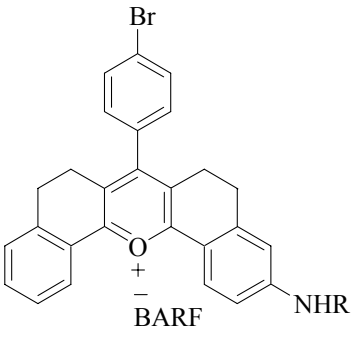
Diamonopyrylium salt (46). 6-Acetamido-2-benzylidene-1-tetralone **45** (0.8 g, 2.16 mmol) and 6-acetamidotetralone **43** (0.41 g, 2.02 mmol) were heated at 100 ^{o}C with 4 mL of boron trifluoride-ether with stirring for 24 hours. The reaction mixture was then cooled to room temperature and then stirred with Et_2O. The product was filtered and washed with Et_2O to give 1.3 g of diaminopyrylium tetrafluoroborate was obtained. To a solution of crude product in CH_2Cl_2/H_2O was added sodium *tetra*(3,5-trifluoromethylbenzene)boronate (NaBARF) (4.1 g, 4.6 mmol). The mixture was stirred at room temperature for 12 hours. The mixture was then extracted with 25 % *iso*propanol in chloroform, and the organic extract was washed with H_2O. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude product was adsorbed on silica, then purified by flash chromatography eluting with 50 % EtOAc/Hexanes, but **46** could not be isolated because of the unavailability of

HPLC to purify it, so that the ^{1}H-NMR and ^{13}C-NMR taken were messy; mp not taken because the compound was impure; MS (ESI): m/z 469/471 M^{+}.



48

2-Benzylidene-1-tetralone (48). In the same manner as for **45**, the title compound was prepared by using tetralone **47** (3.7 g, 25.3 mmol), 4-bromo-benzaldehyde (4.7 g, 25.3 mmol), and 3 mL of 4 % ethanolic potassium hydroxide. The crude product was then recrystallized from ethanol to give **48** (6.3 g, 80 %) as orange crystals: ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 2.98 (t, *J* = 6.9 Hz, 2H), 3.12 (t, *J* = 6.9 Hz, 2H), 7.30-7.60 (m,7H), 7.81 (s, 1H), 8.16 (dd, *J* = 6.3 Hz, 1.5 Hz, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) 27.45, 29.03, 122.99, 127.38, 128.48, 128.53, 131.63 (2 CH), 131.94 (2 CH), 133.59, 133.69, 134.97, 135.55, 136.33, 143.41, 187.92. Hammam, A. E.; Hussain, M.; Kotob, I. R. *Phosphorus, Sulfur, and Silicon* **1990**, *47*, 47-51.



R = H, Ac

49

Monoaminopyrylium salt (48). In the same manner as for **46**, the title compound was prepared by using 2-benzylidene-1-tetralone **48** (0.81 g 2.58 mmol), 6-

acetamidotetralone **43** (0.5 g, 2.46 mmol), boron trifluoride-ether (5 mL, 40 mmol), and NaBARF (4.9 g, 5.54 mmol) to give 1.5 g of monoaminopyrylium tetrafluoroborate. The crude product was adsorbed on silica, then purified by flash chromatography eluting with, but **49** could not be isolated either because of the unavailability of HPLC to purify it, so that the ^{1}H-NMR taken was messy; mp not taken because the compound was impure; MS (ESI): m/z 454/456 M^{+}.

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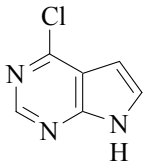
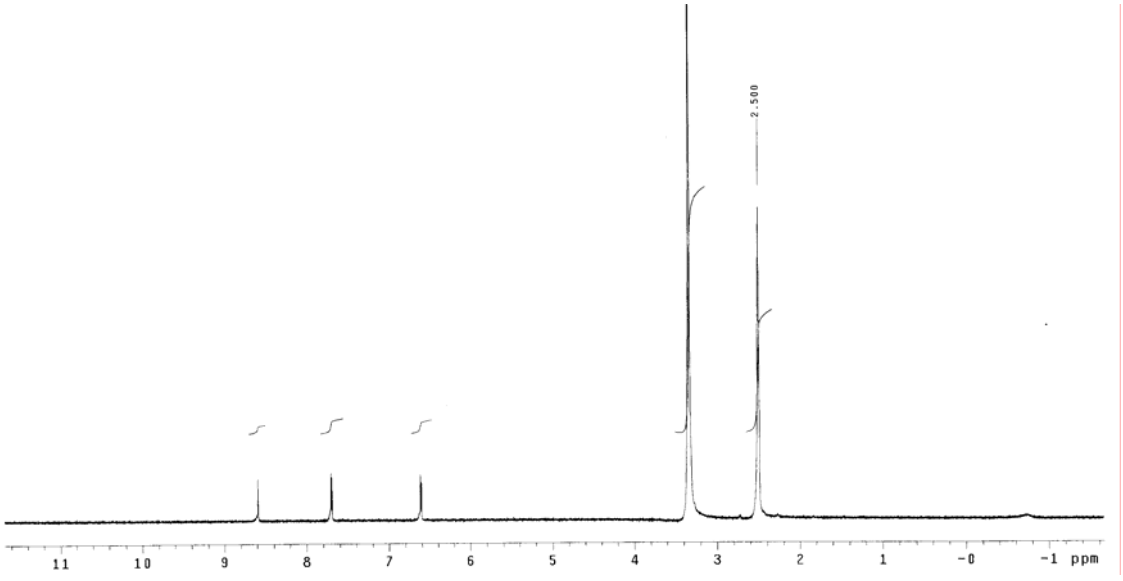
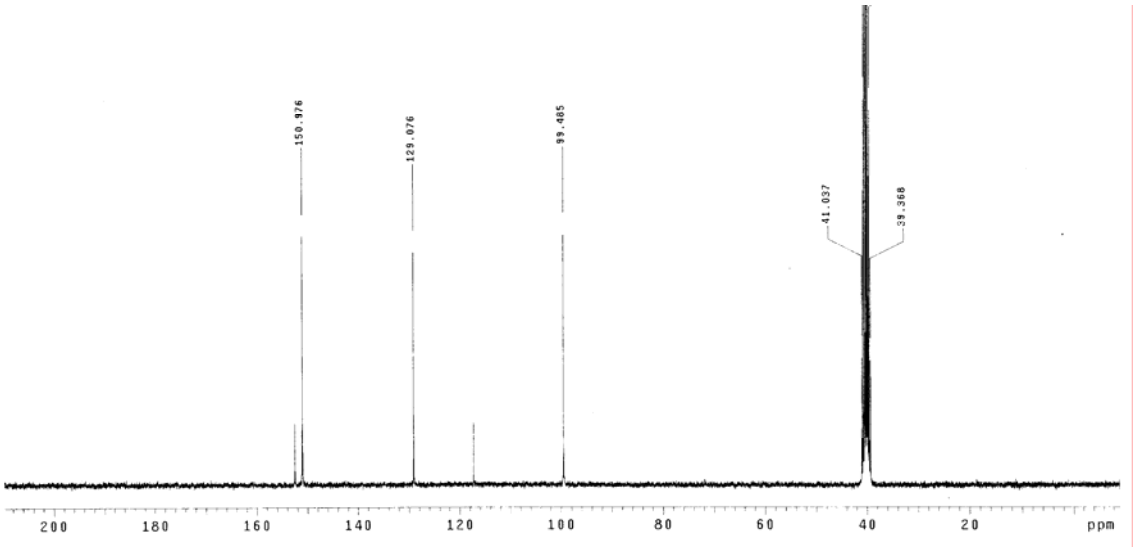
APPENDIX I

SELECTED SPECTRA



7

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60
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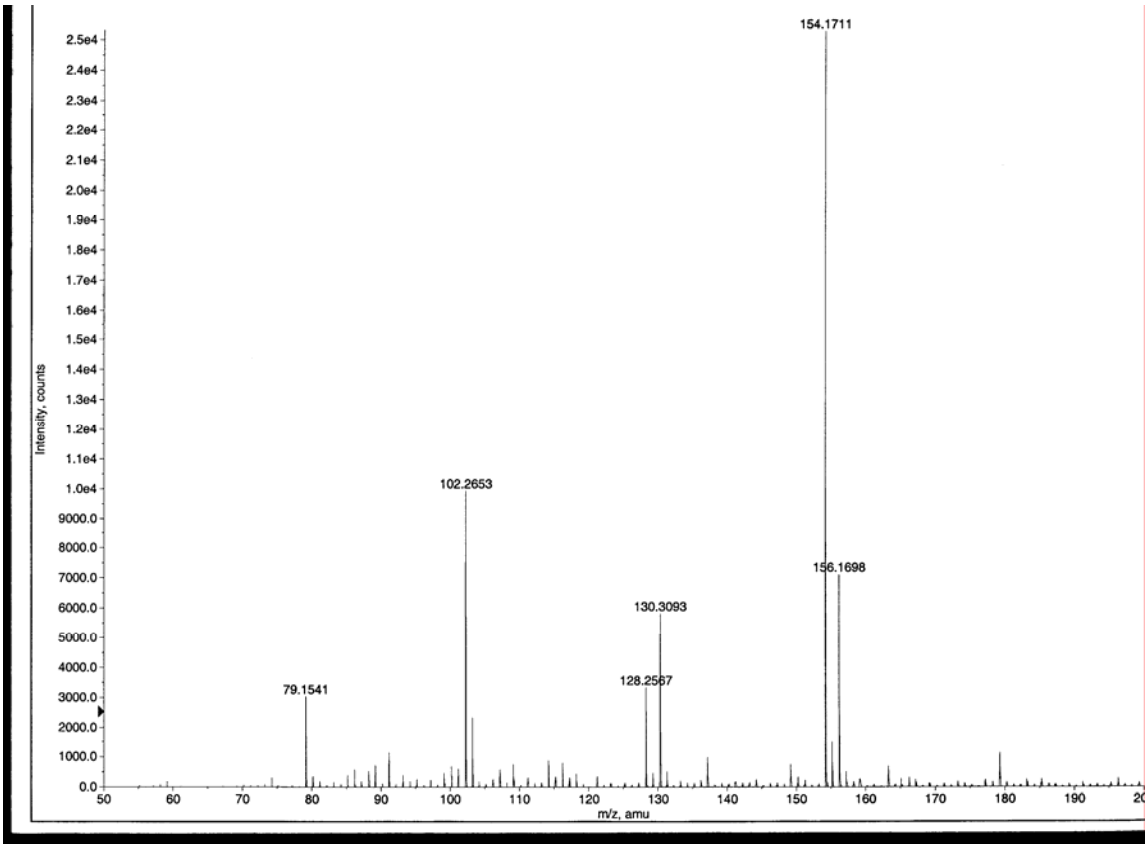
ppm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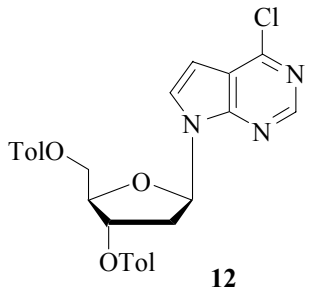

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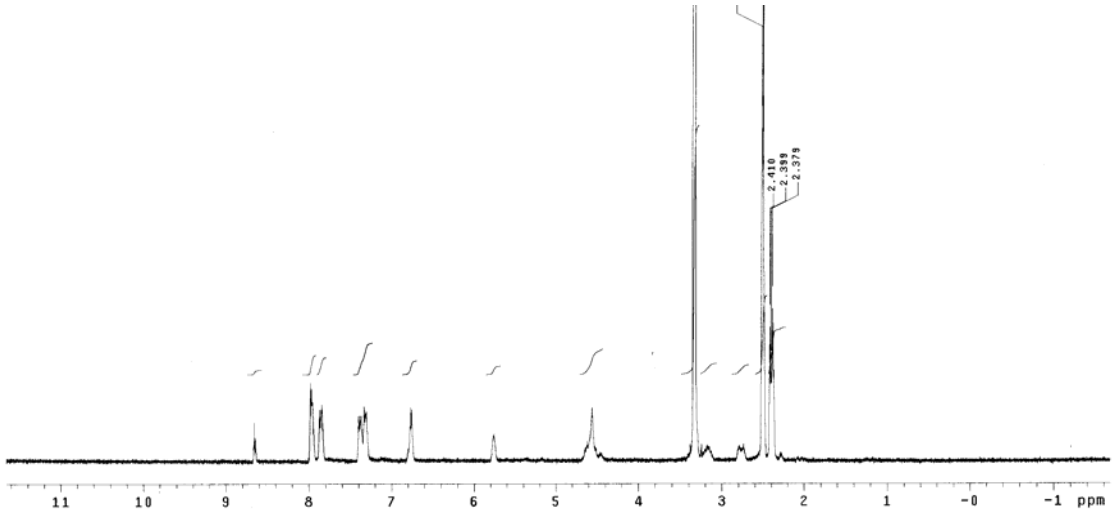

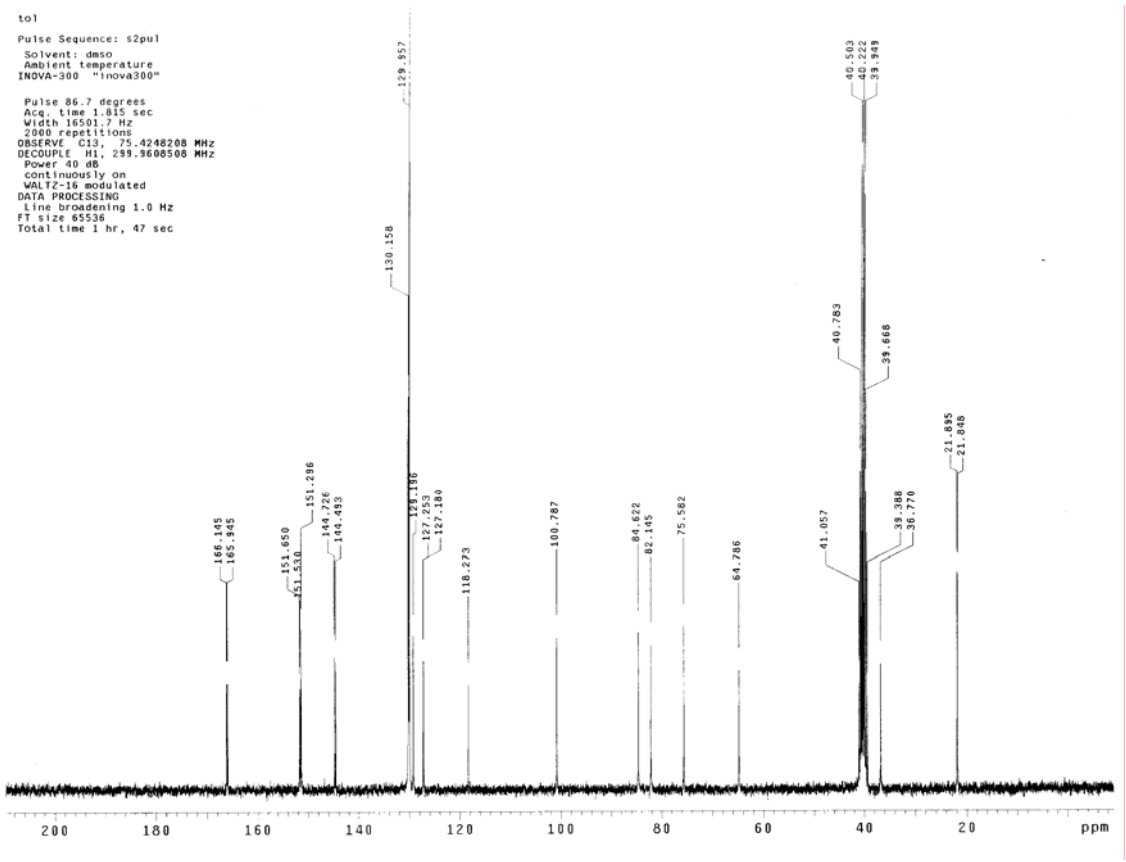
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2000 repetitions
OBSERVE C13, 75.4248208 MHz
DECOUPLE H1, 299.9608508 MHz
Power 40 dB
continuously on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 1.0 Hz
FT size 65536
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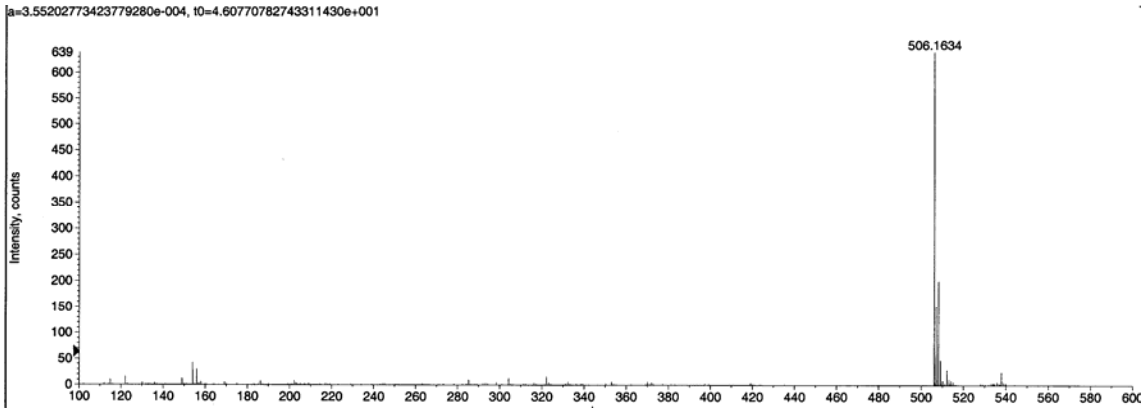
ppm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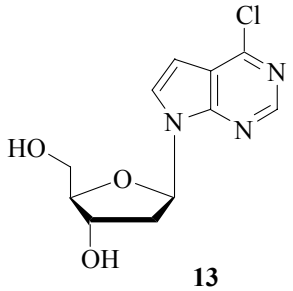
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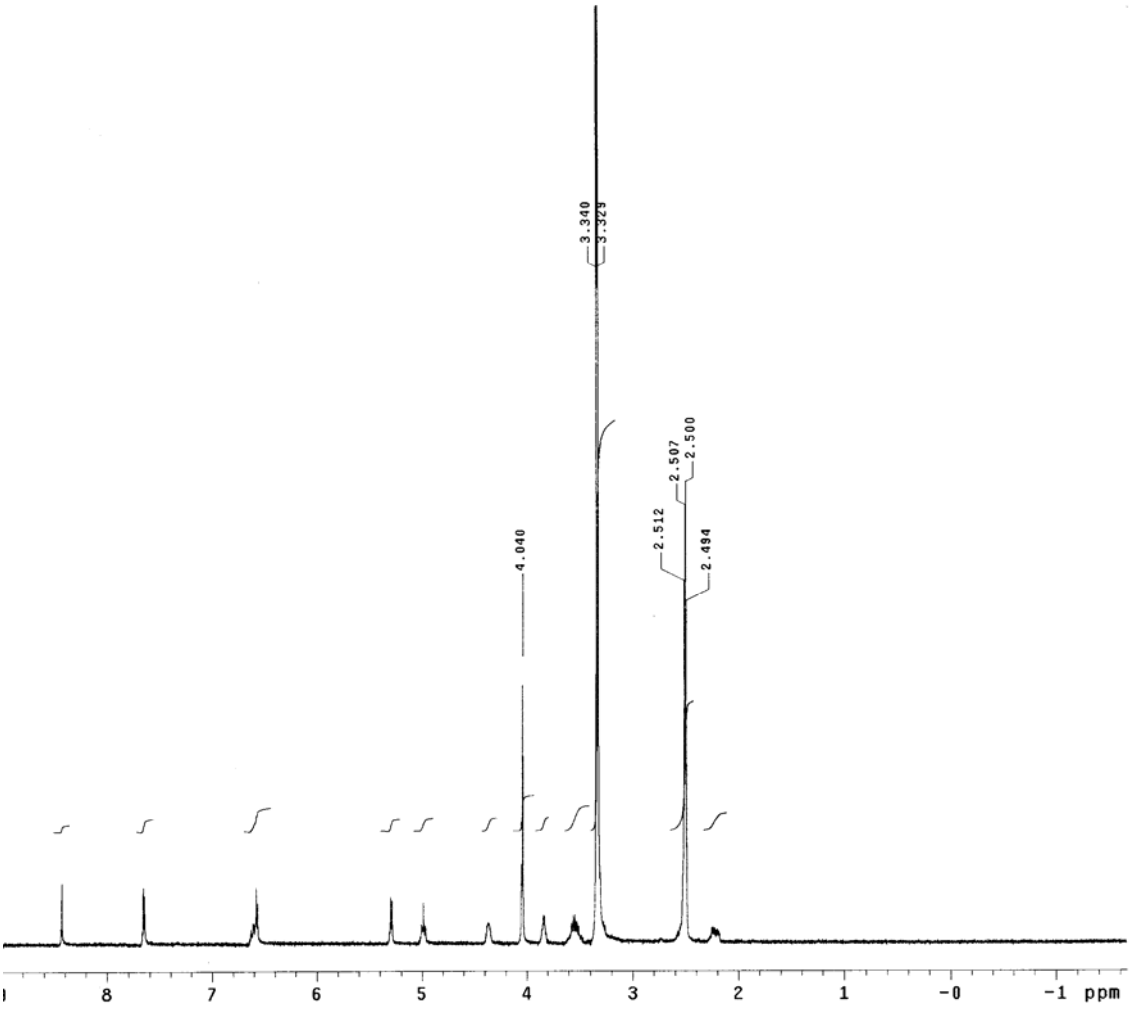
13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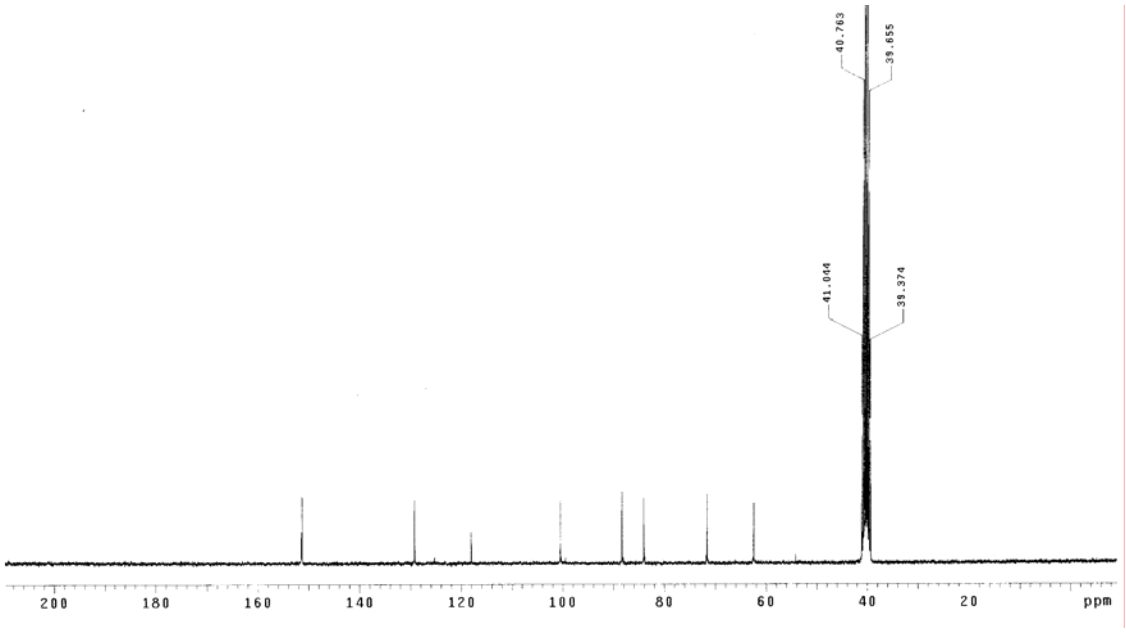
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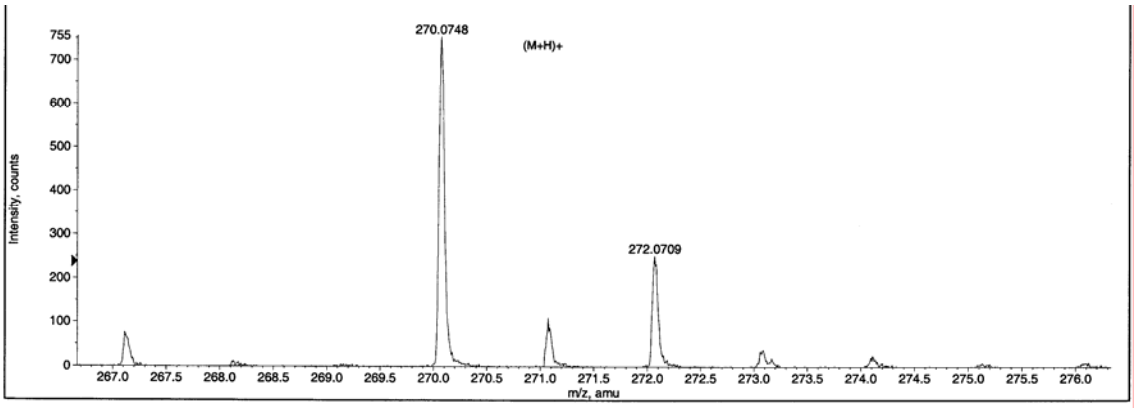
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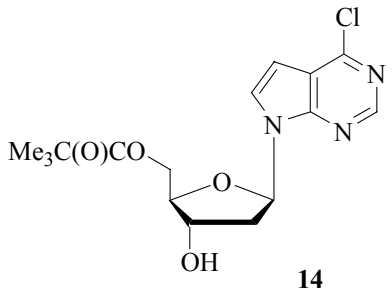
Intensity, counts
m/z, amu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Me3C(O)CO
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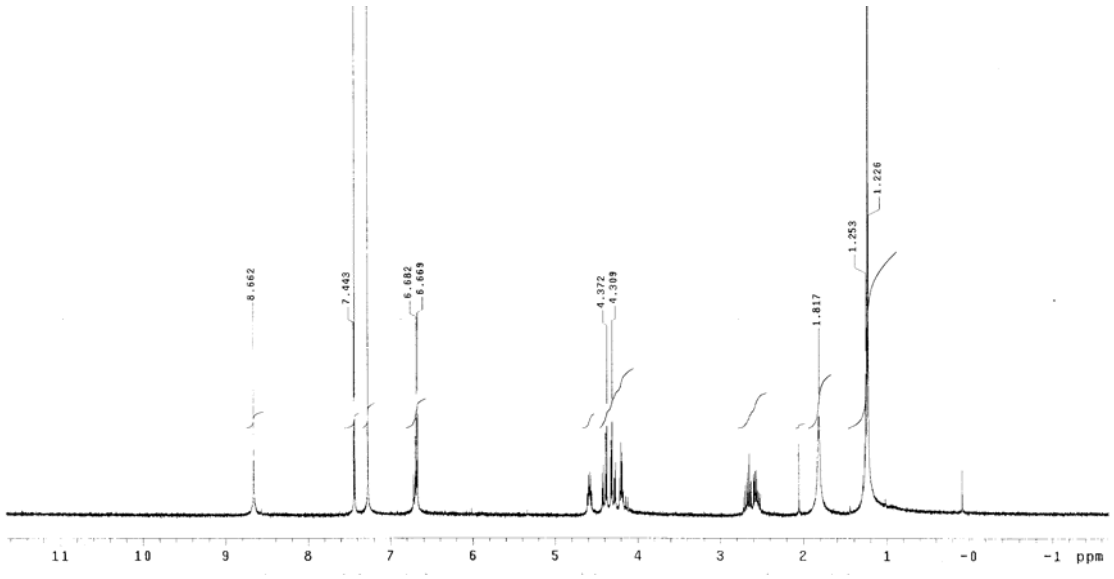
OH

14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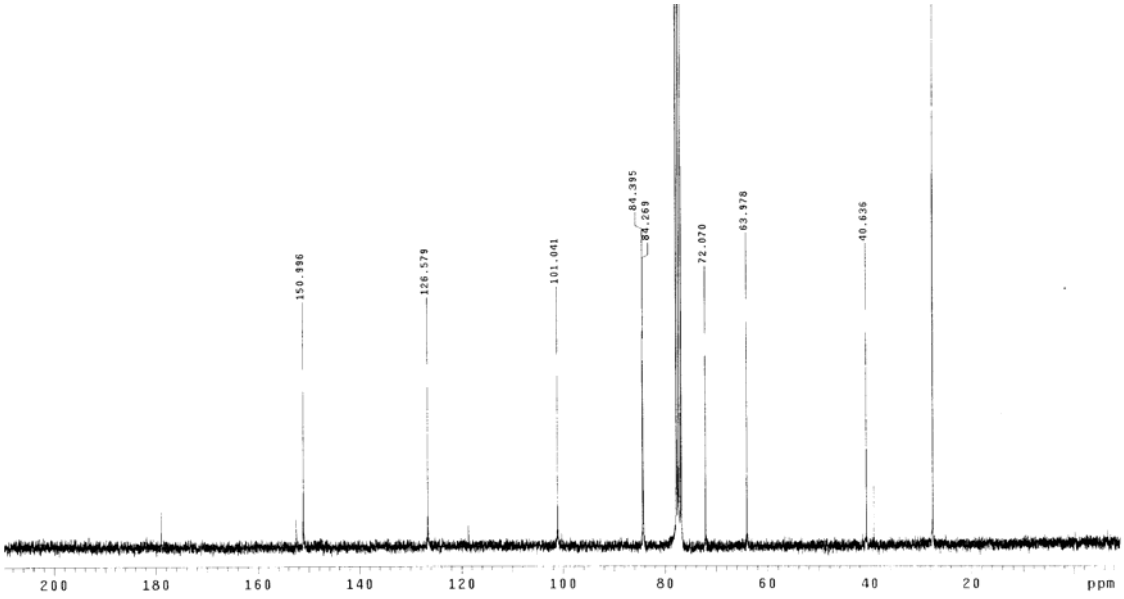
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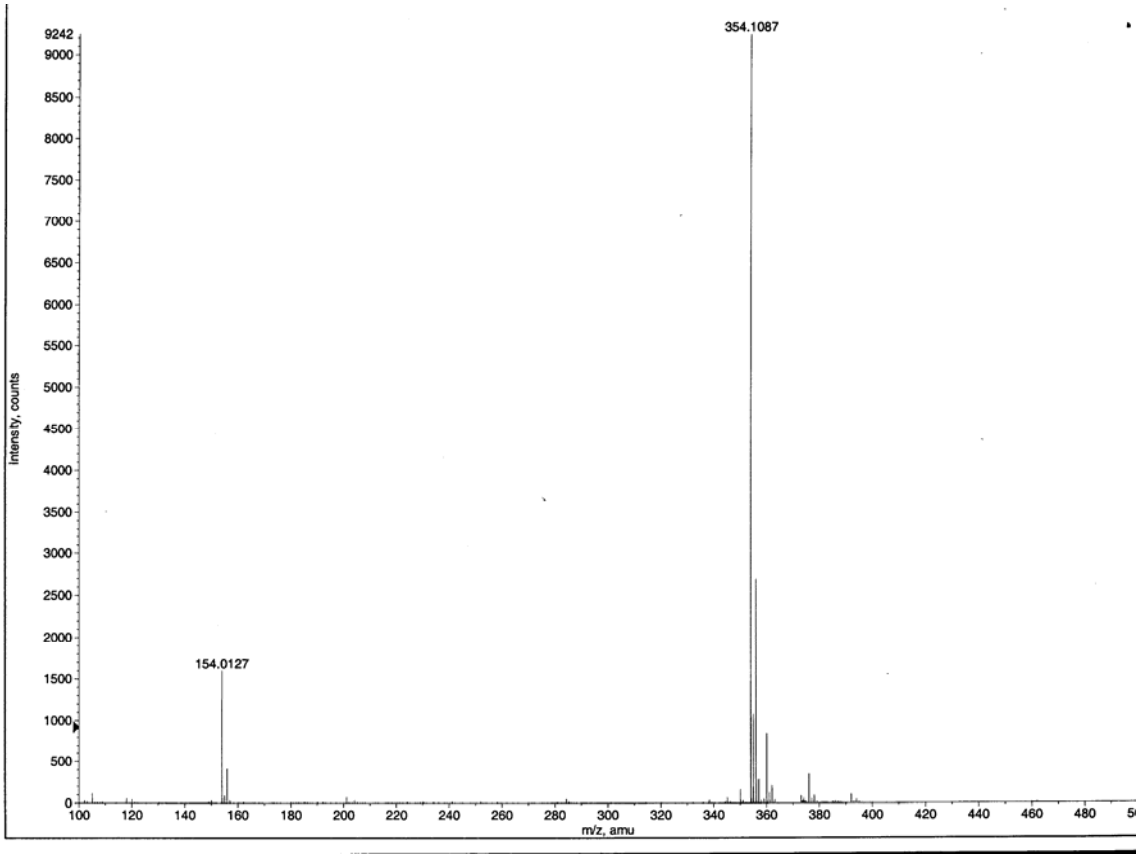
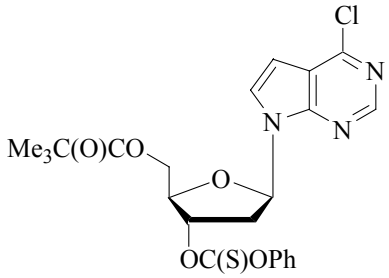
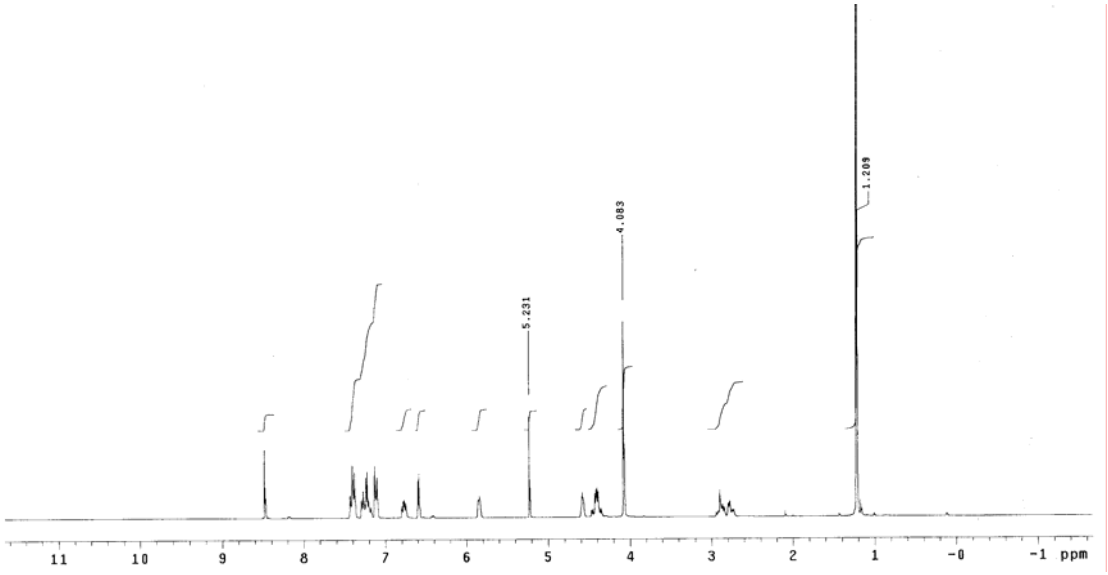
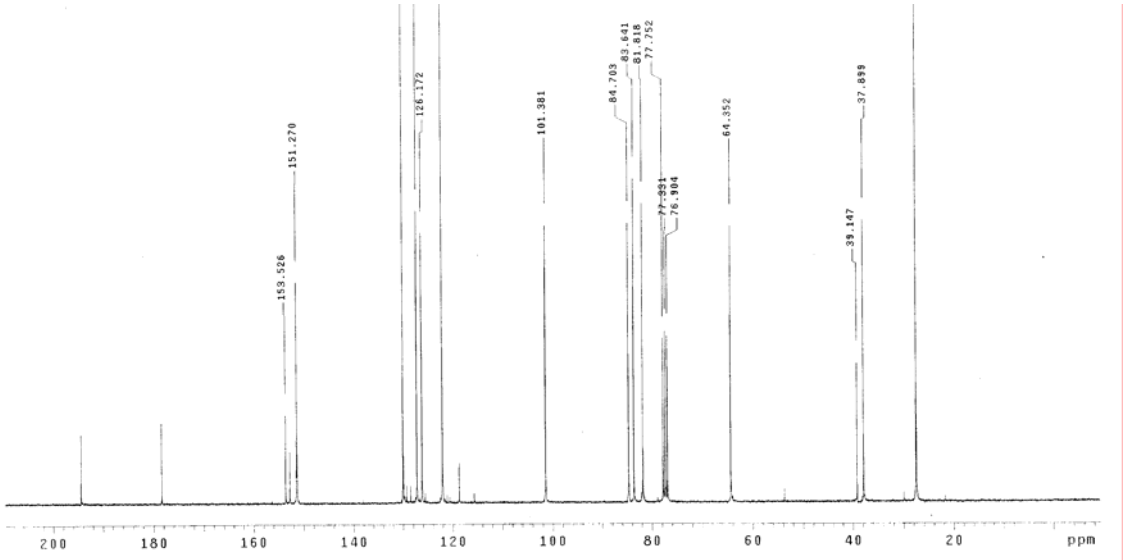
20
ppm

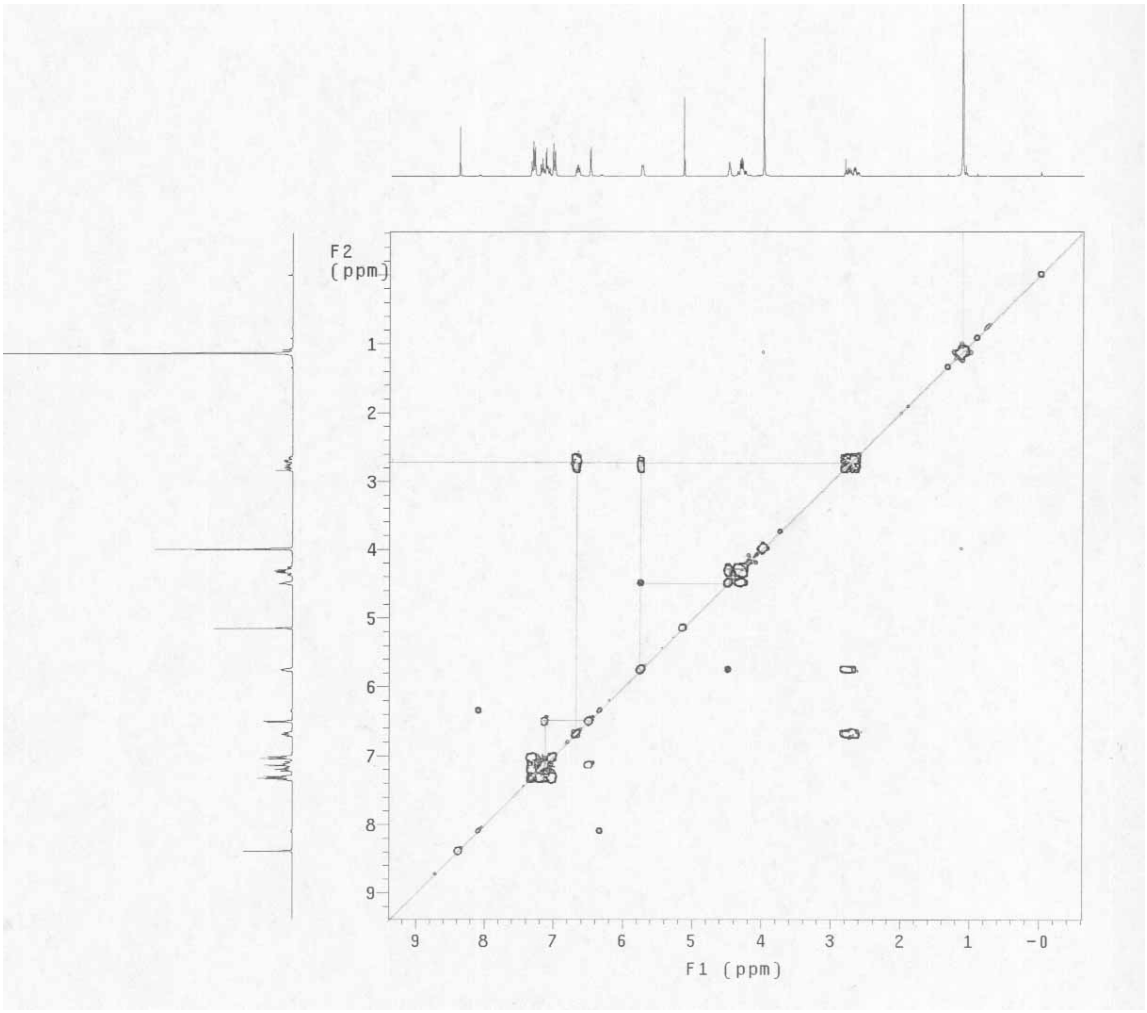

354.1087
154.0127
Intensity, counts
m/z, amu
9242
9000
8500
8000
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480

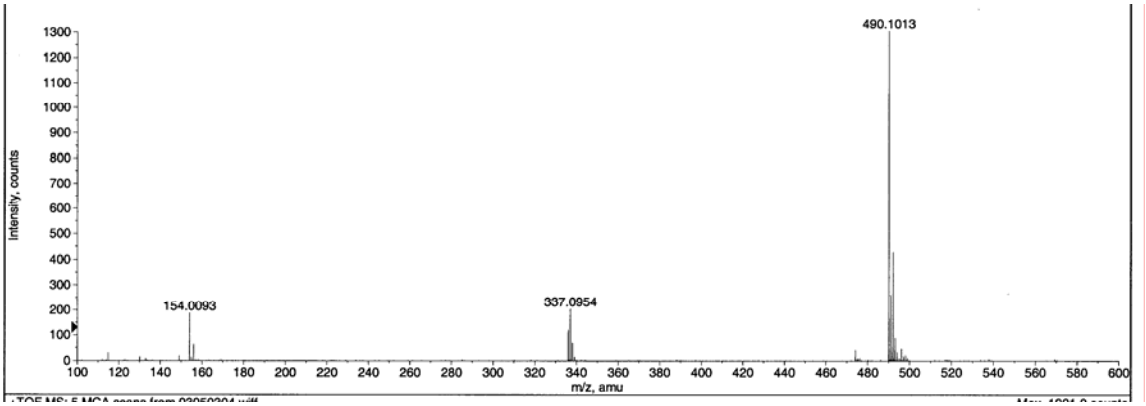

Cl
N
N
N
Me3C(O)CO
O
OC(S)OPh

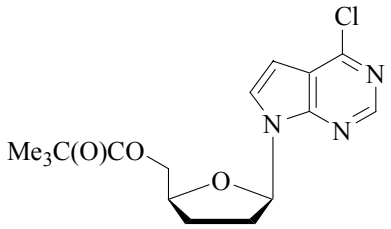
15


1.209
4.083
5.231
11
10
9
8
7
6
5
4
3
2
1
-0
-1 ppm

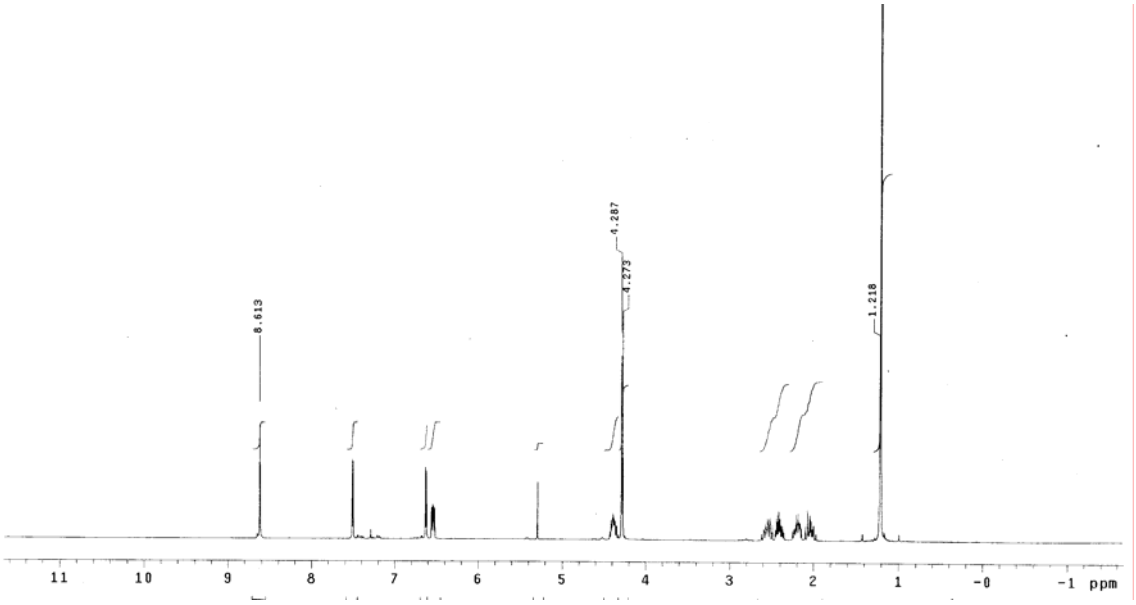

153.526
151.270
126.172
101.381
84.703
83.641
81.818
77.752
77.001
76.904
64.352
39.147
37.859
200
180
160
140
120
100
80
60
40
20
ppm

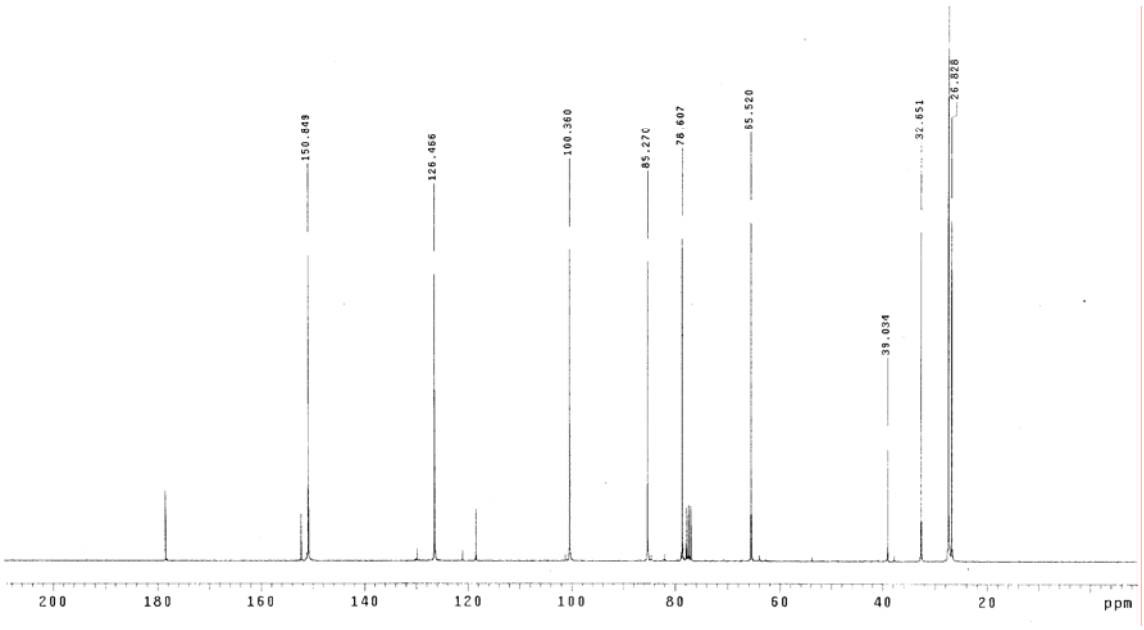

F2
(ppm)
F1 (ppm)

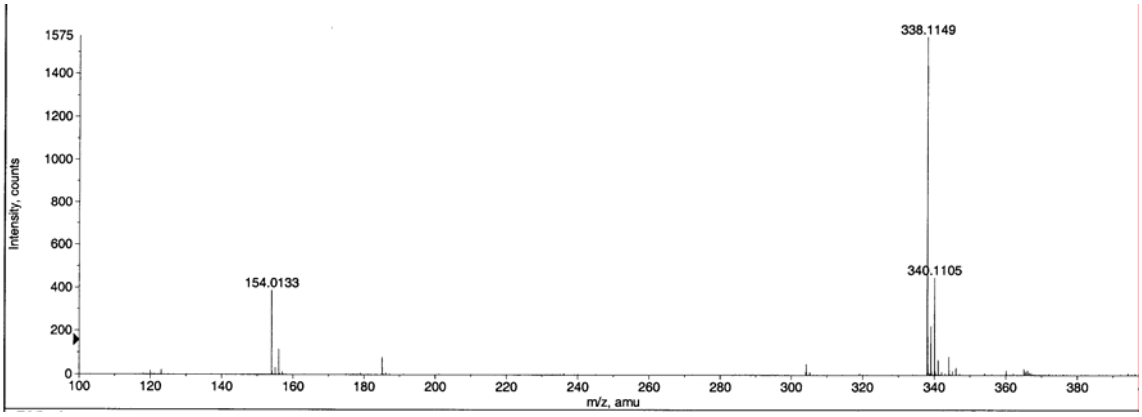

Intensity, counts
m/z, amu
490.1013
337.0954
154.0093

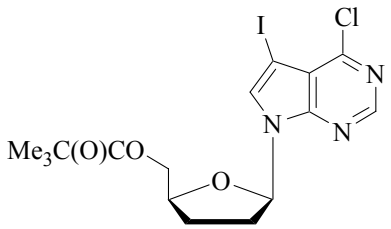


16

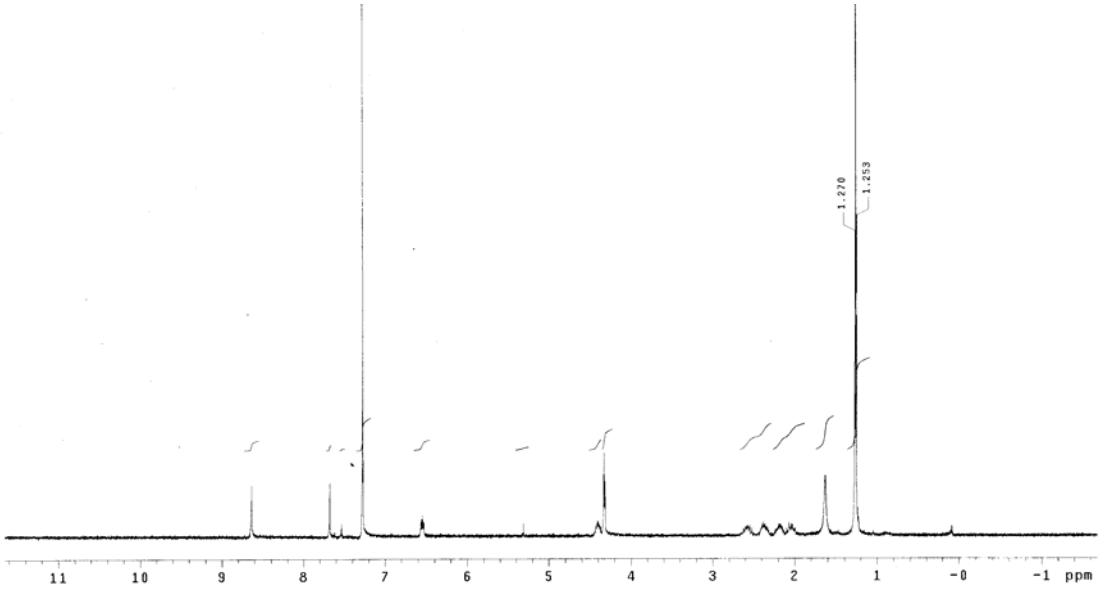


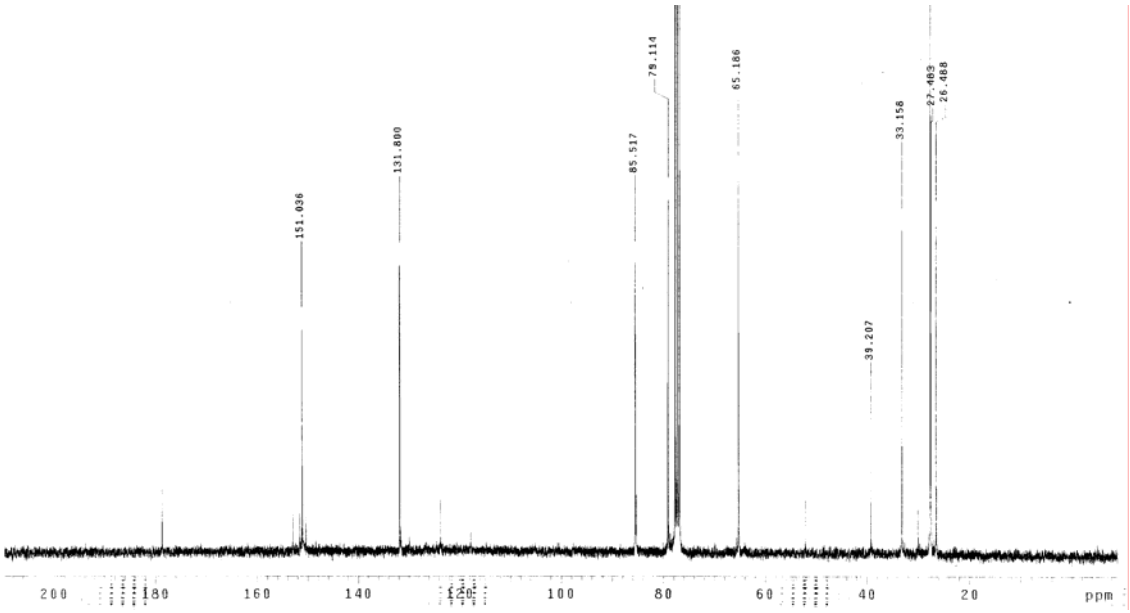

150.849
126.466
100.360
85.270
78.607
65.520
39.034
32.651
26.828
200
180
160
140
120
100
80
60
40
20
ppm

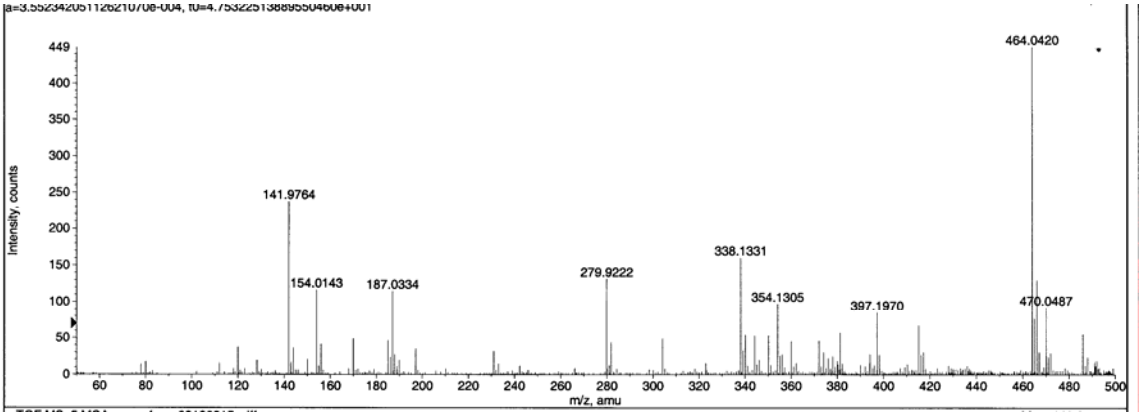

Intensity, counts
1575
1400
1200
1000
800
600
400
200
0
338.1149
340.1105
154.0133
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
m/z, amu

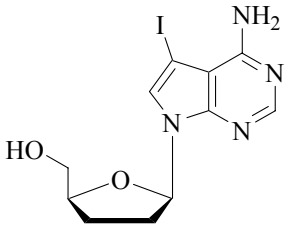


17

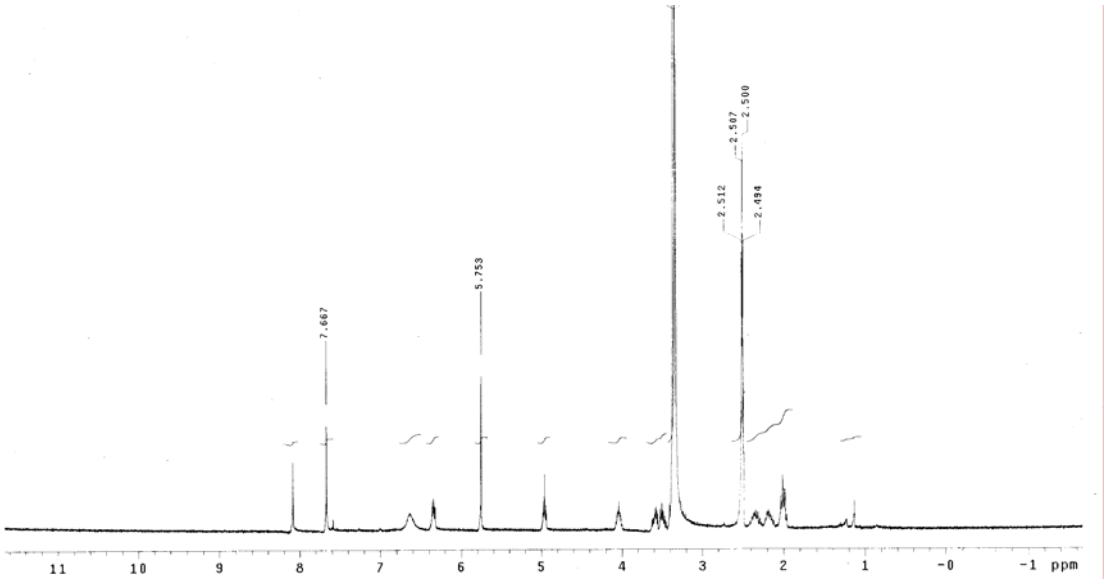


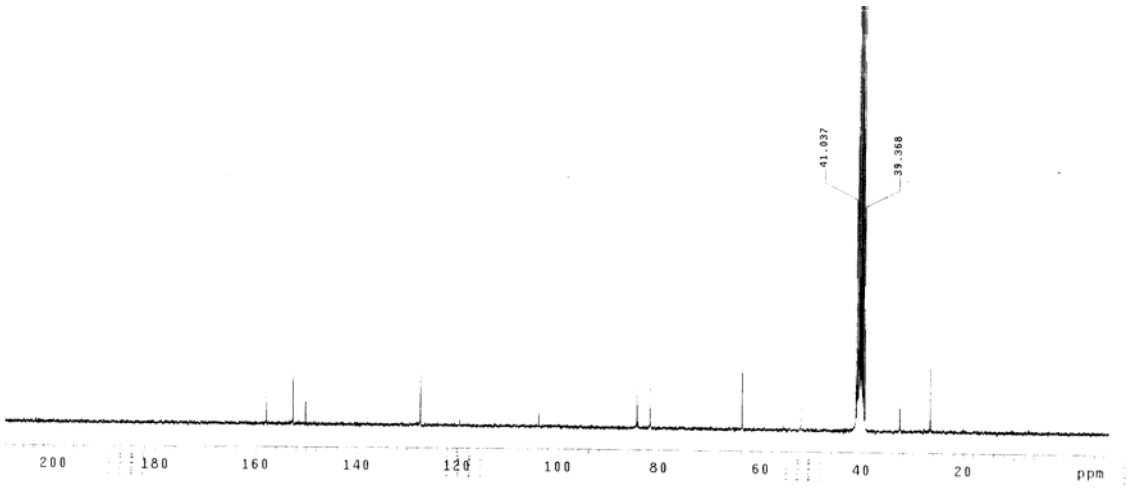

151.036
131.800
85.517
79.114
65.186
39.207
33.158
27.463
26.488
200
180
160
140
120
100
80
60
40
20
ppm

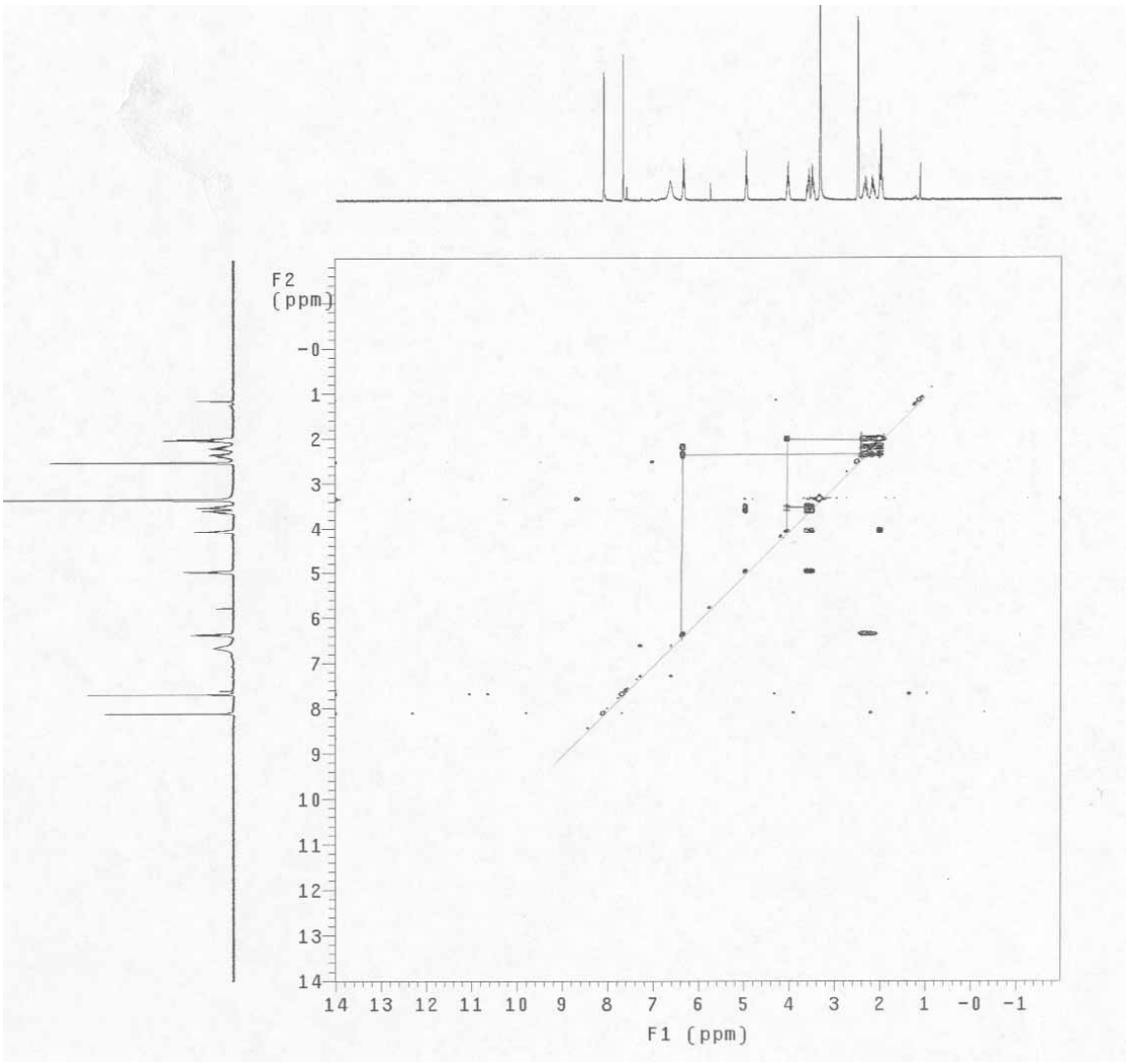

Intensity, counts
m/z, amu
464.0420
141.9764
154.0143
187.0334
279.9222
338.1331
354.1305
397.1970
470.0487

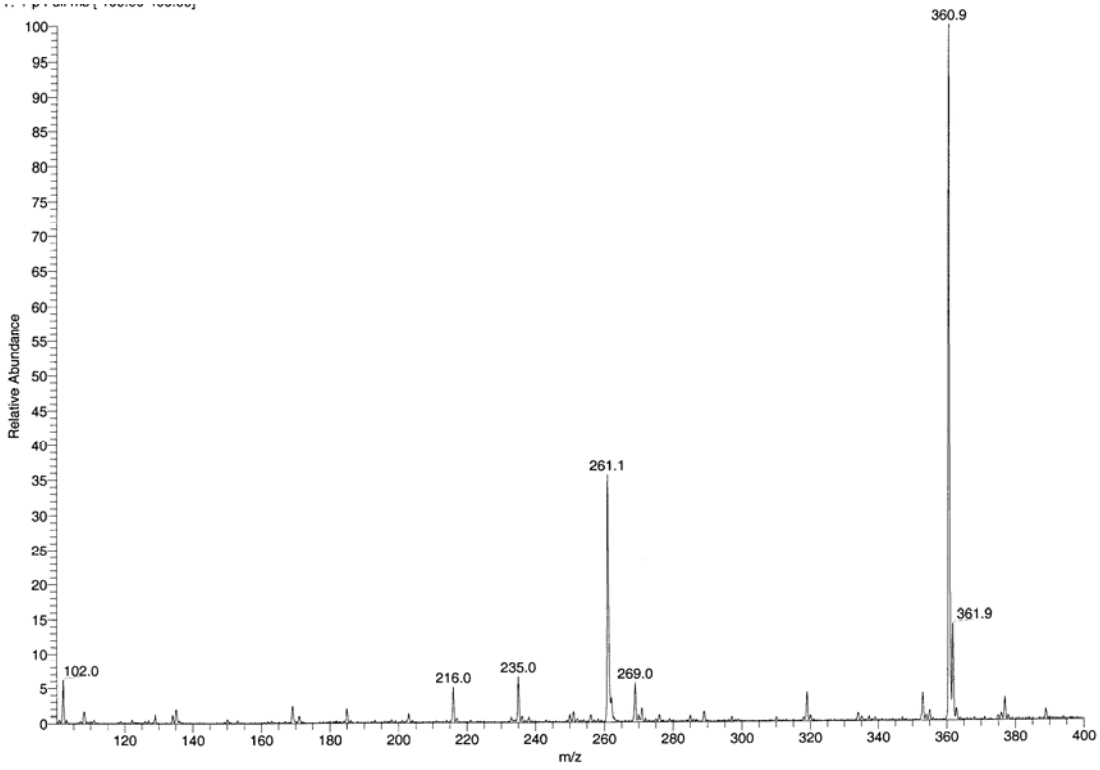

I
NH2
N
N
N
HO
O

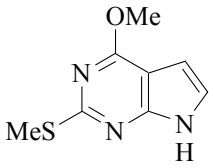
18


7.667
5.753
2.512
2.507
2.500
2.494
11
10
9
8
7
6
5
4
3
2
1
-0
-1 ppm

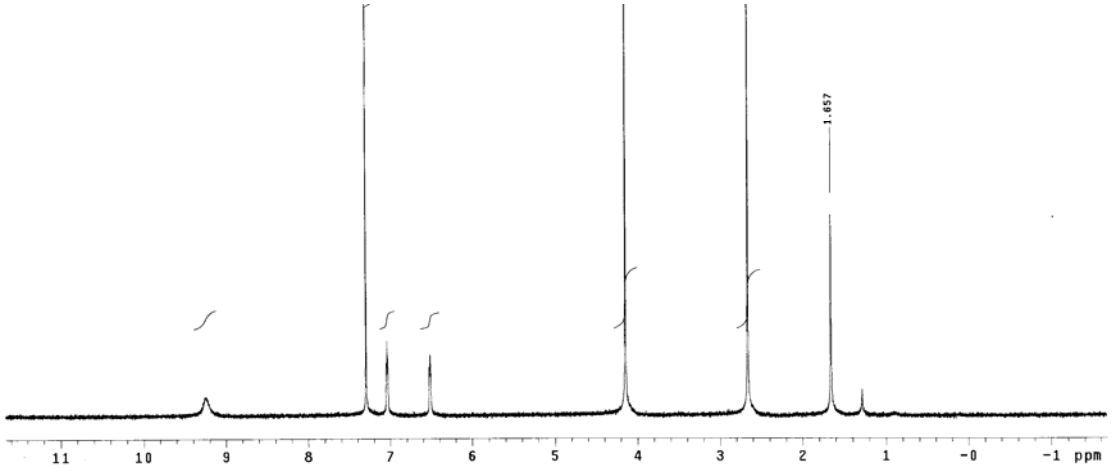

41.037
39.368
200
180
160
140
120
100
80
60
40
20
ppm

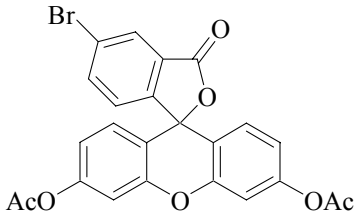

F2
(ppm)
-0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
14 13 12 11 10 9 8 7 6 5 4 3 2 1 -0 -1
F1 (ppm)


360.9
261.1
361.9
102.0
216.0
235.0
269.0
Relative Abundance
m/z

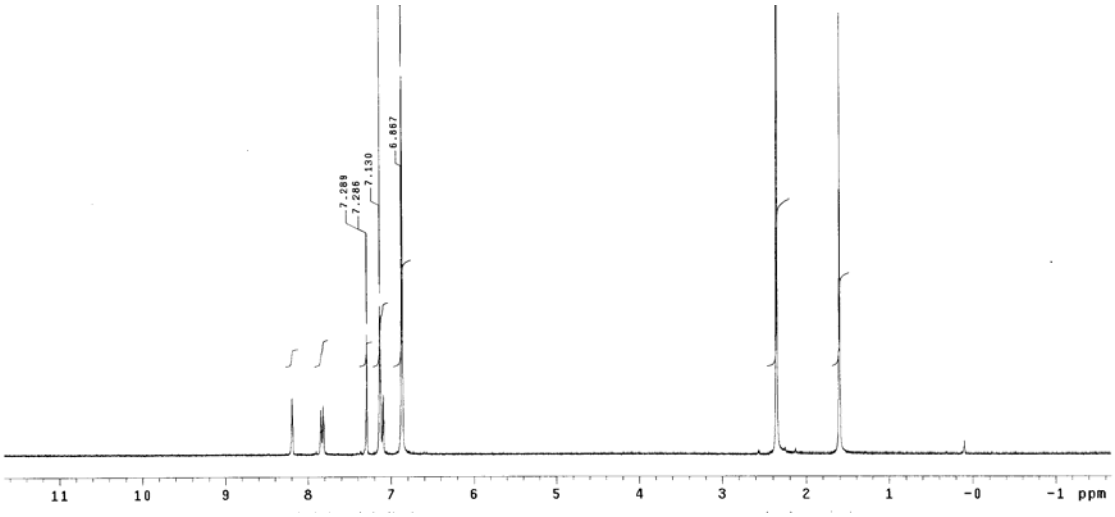


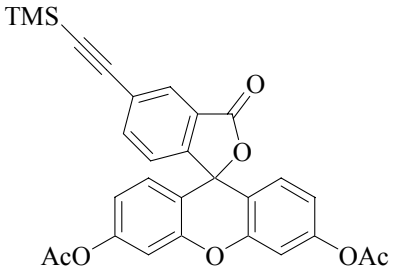
22



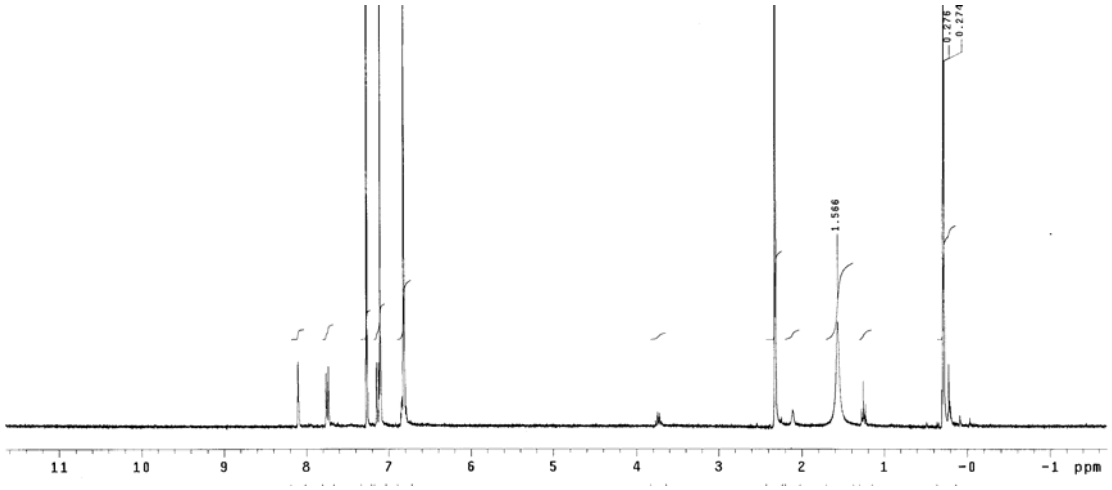


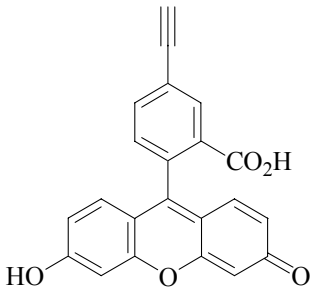
26



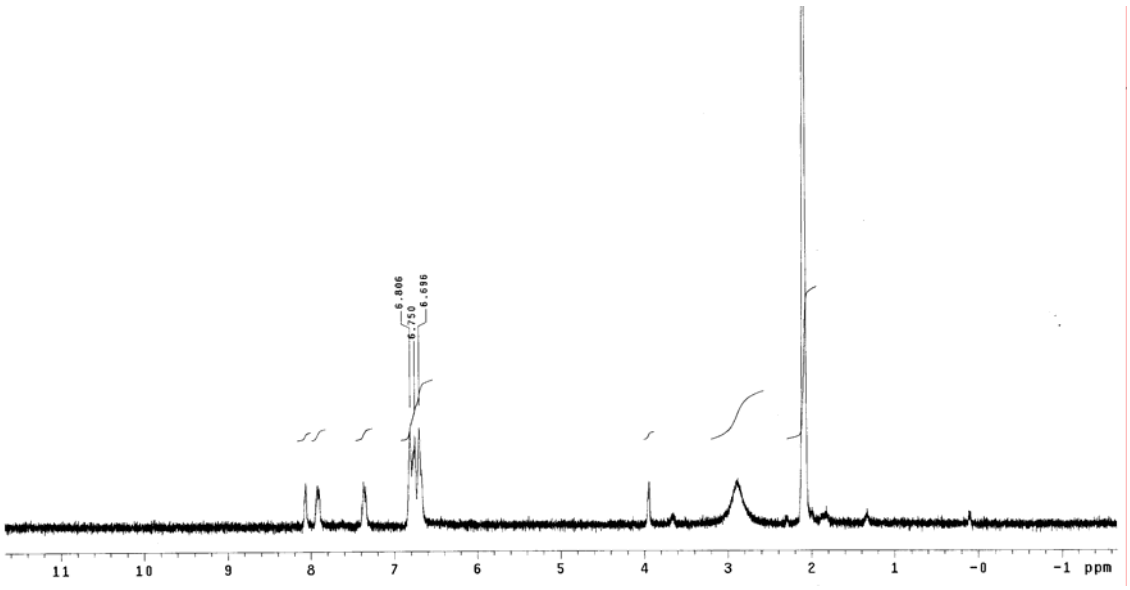


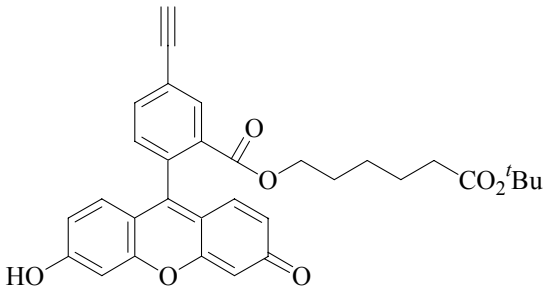
27



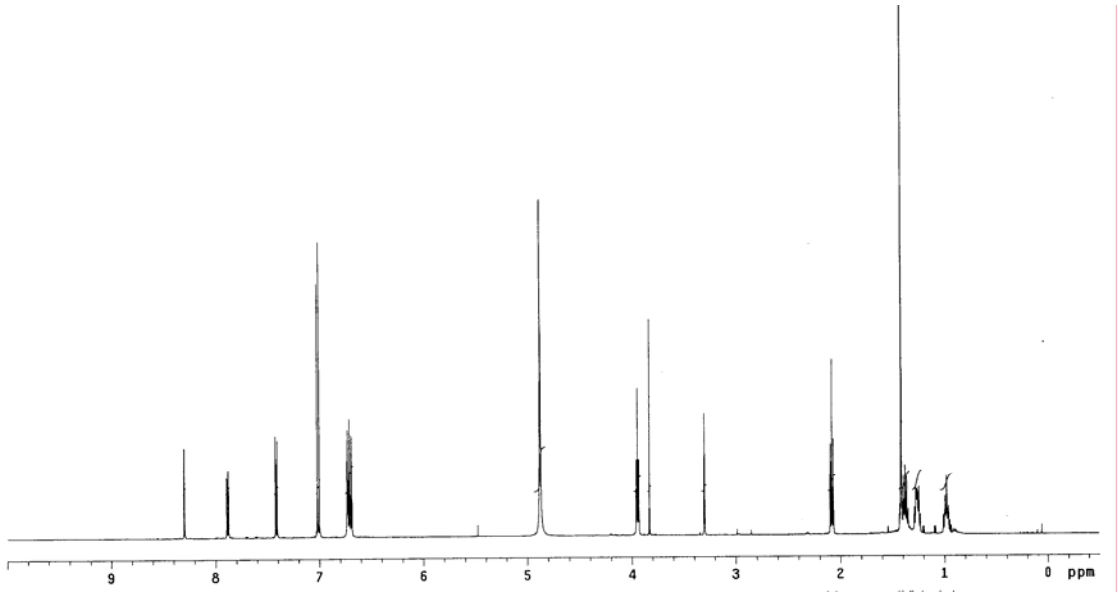


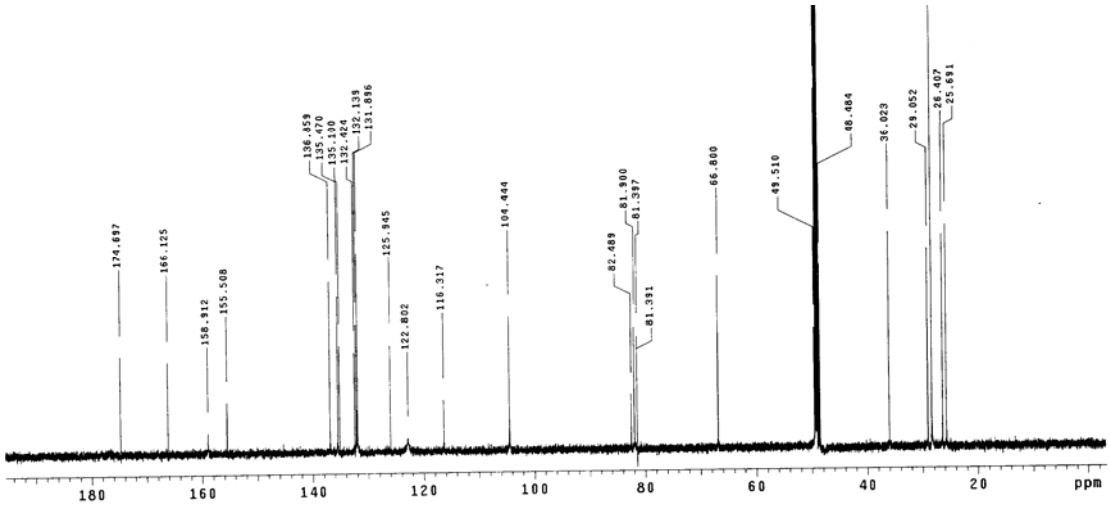
28

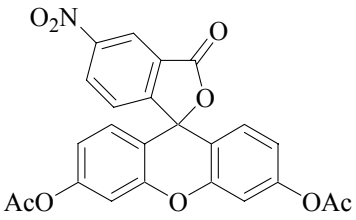



O
O
$CO_2{}^tBu$
HO
O
O

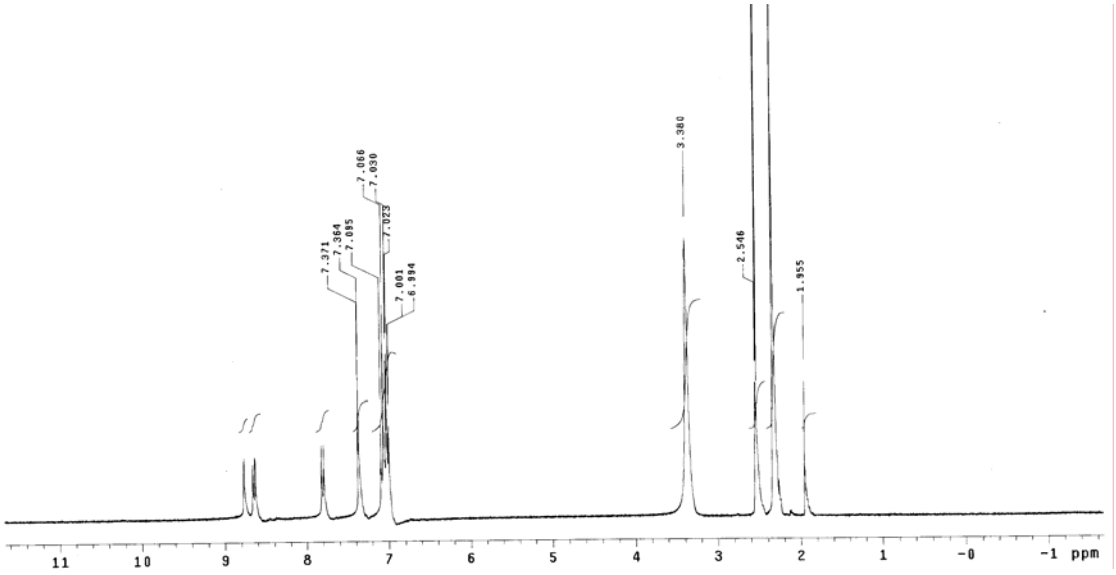
29

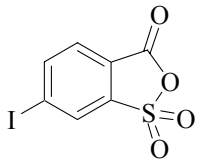

9
8
7
6
5
4
3
2
1
0 ppm


174.697
166.125
158.912
155.508
136.459
135.470
135.100
132.424
132.139
131.896
125.845
122.802
116.317
104.444
82.489
81.900
81.397
81.391
66.800
49.510
48.484
36.023
29.052
28.407
25.691
180
160
140
120
100
80
60
40
20
ppm

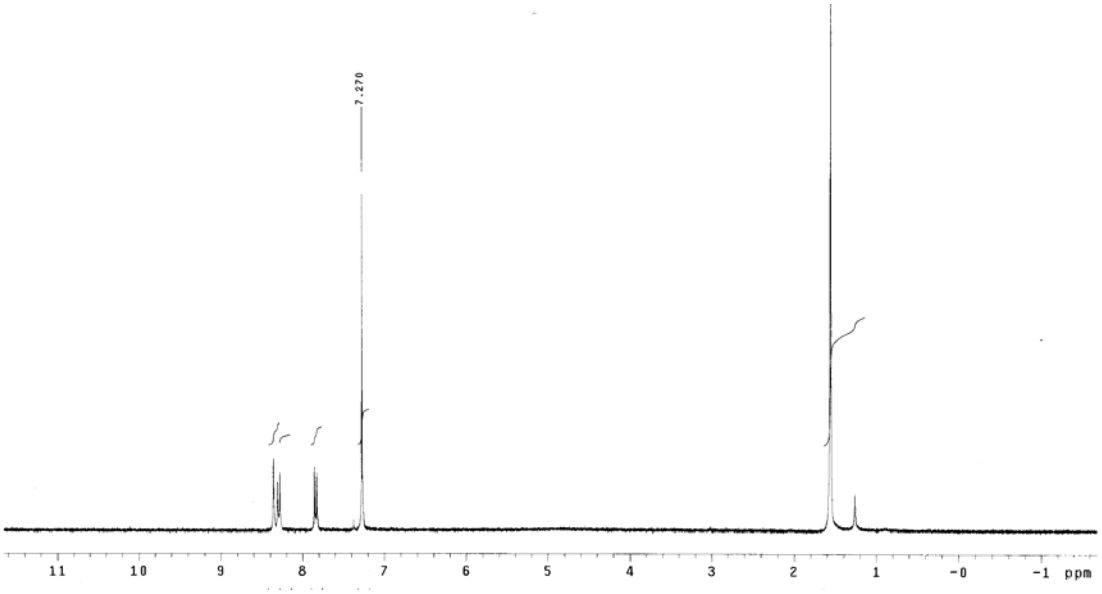

O2N
O
O
AcO
O
OAc

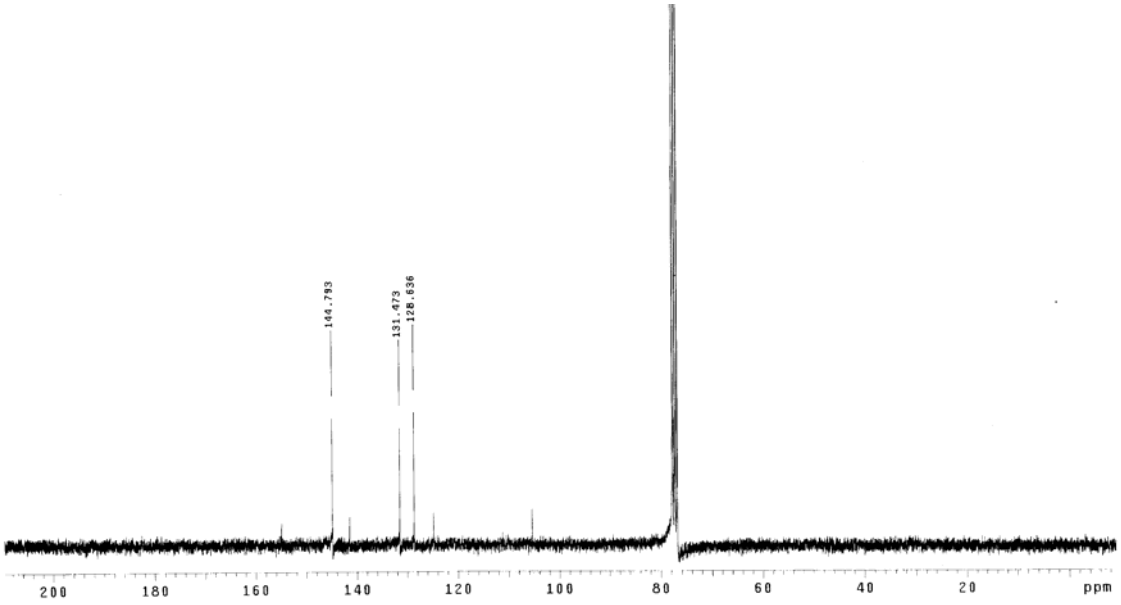
32

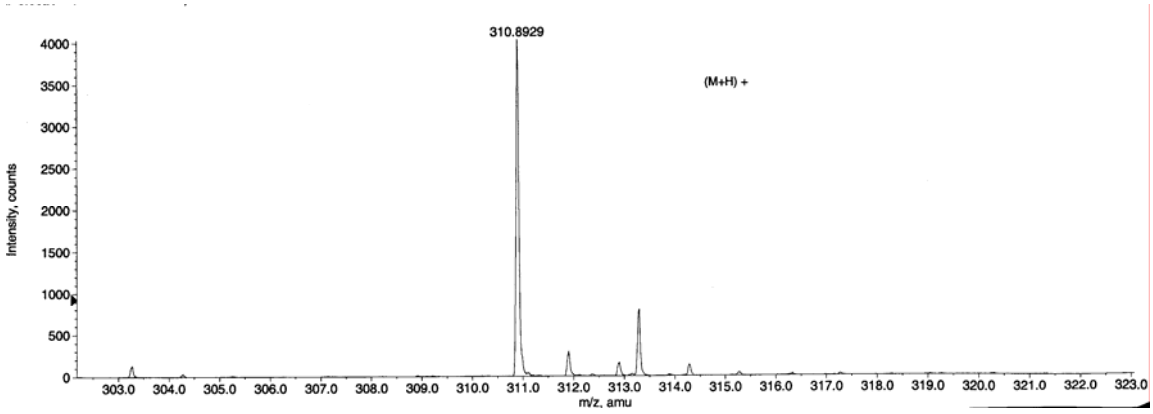

7.371
7.364
7.095
7.066
7.030
7.023
7.001
6.994
3.380
2.546
1.955
11
10
9
8
7
6
5
4
3
2
1
-0
-1 ppm

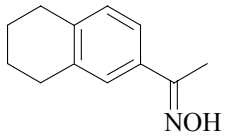


37

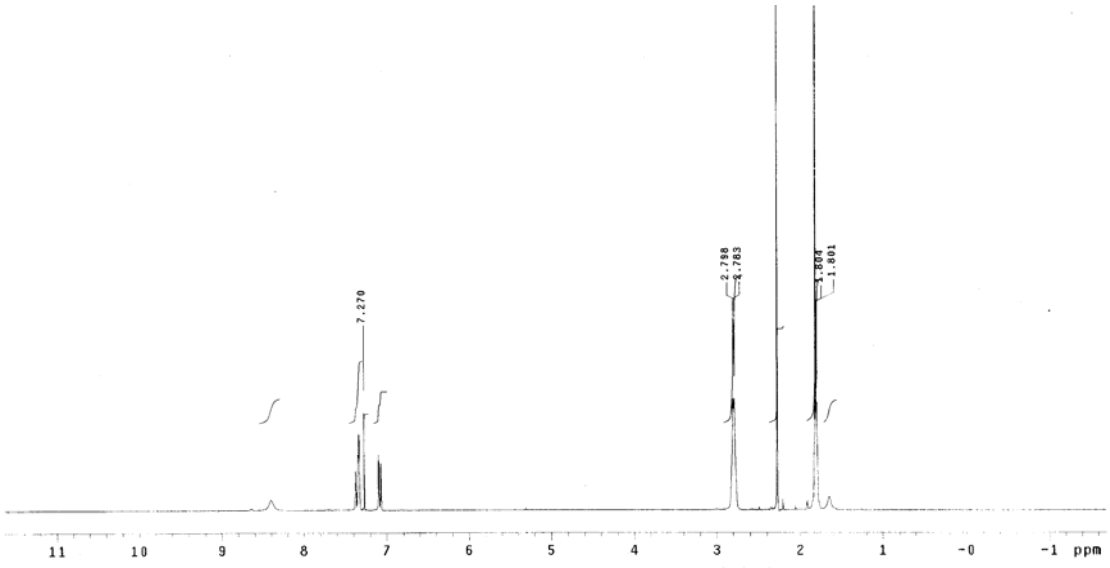


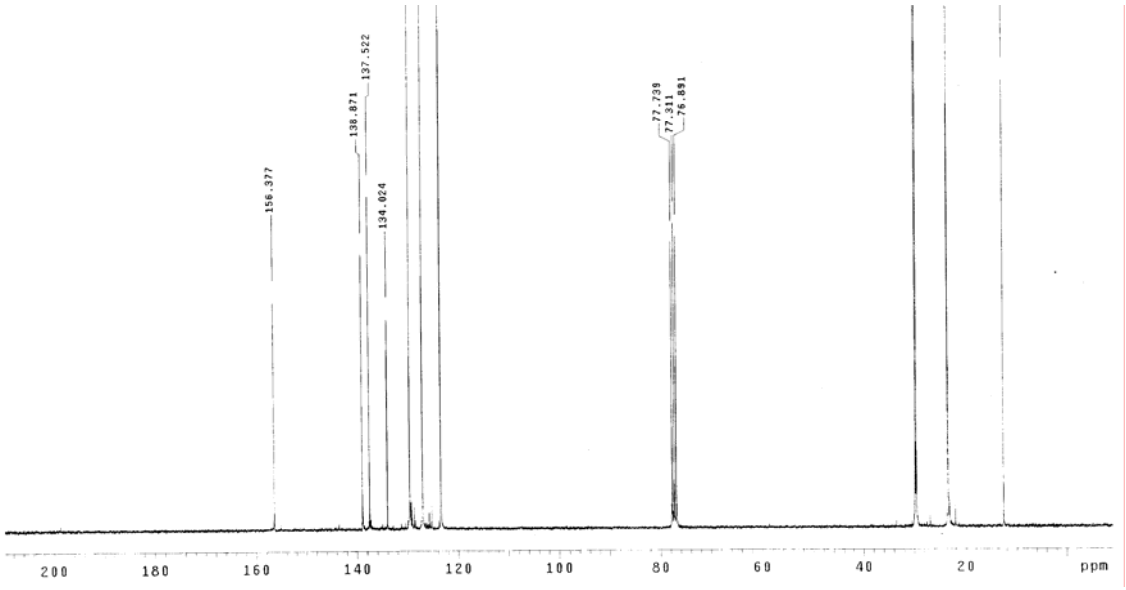

144.793
131.473
128.636
200
180
160
140
120
100
80
60
40
20
ppm

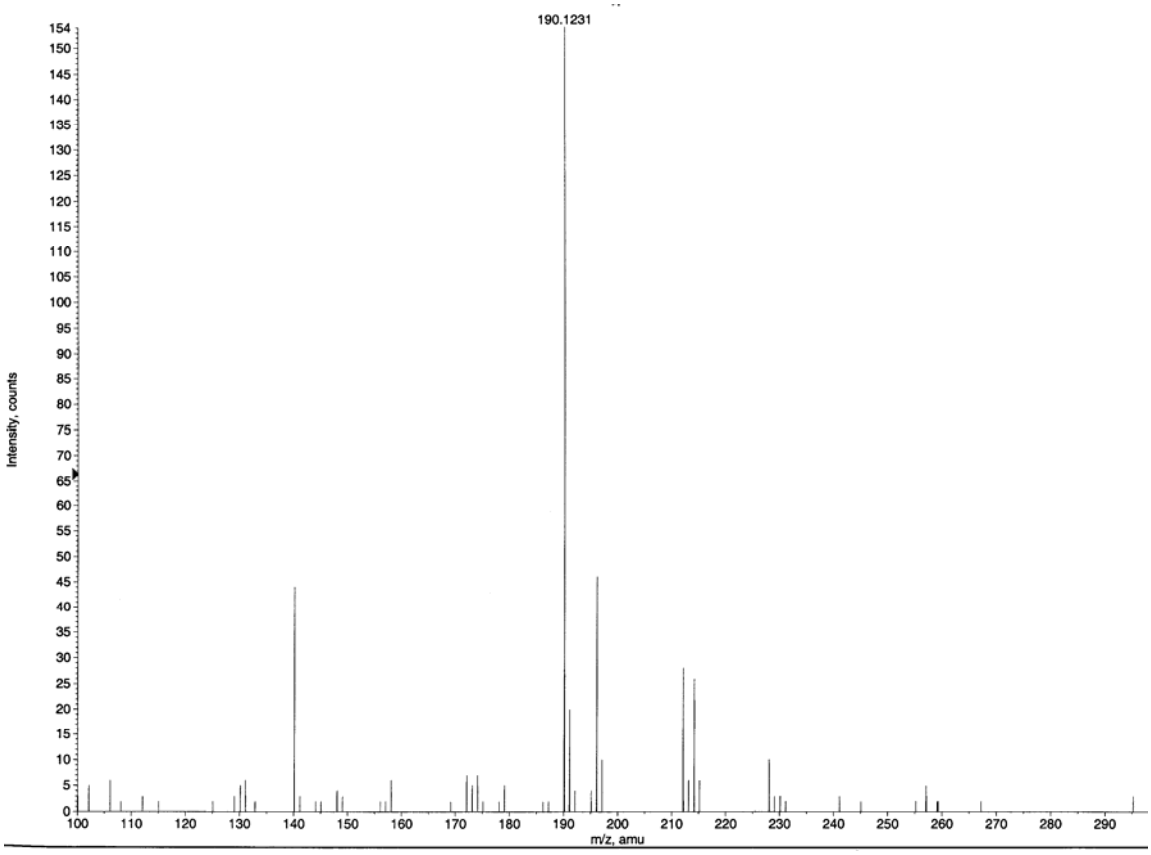

310.8929
(M+H) +
Intensity, counts
4000
3500
3000
2500
2000
1500
1000
500
0
303.0
304.0
305.0
306.0
307.0
308.0
309.0
310.0
311.0
312.0
313.0
314.0
315.0
316.0
317.0
318.0
319.0
320.0
321.0
322.0
323.0
m/z, amu

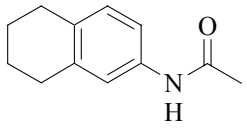

NOH

41

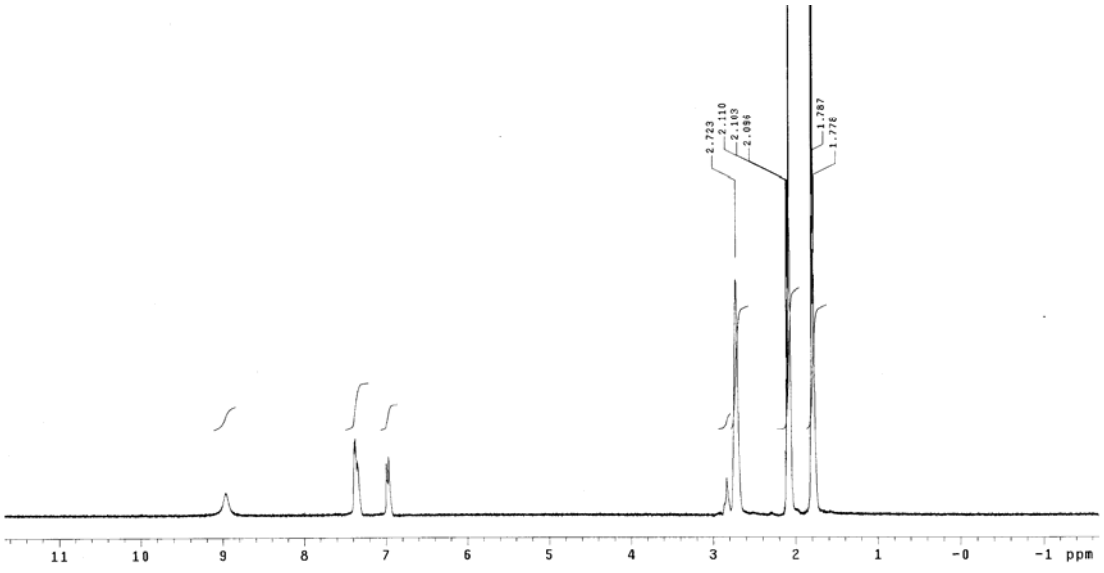

7.270
2.798
2.783
1.804
1.801
11
10
9
8
7
6
5
4
3
2
1
-0
-1
ppm

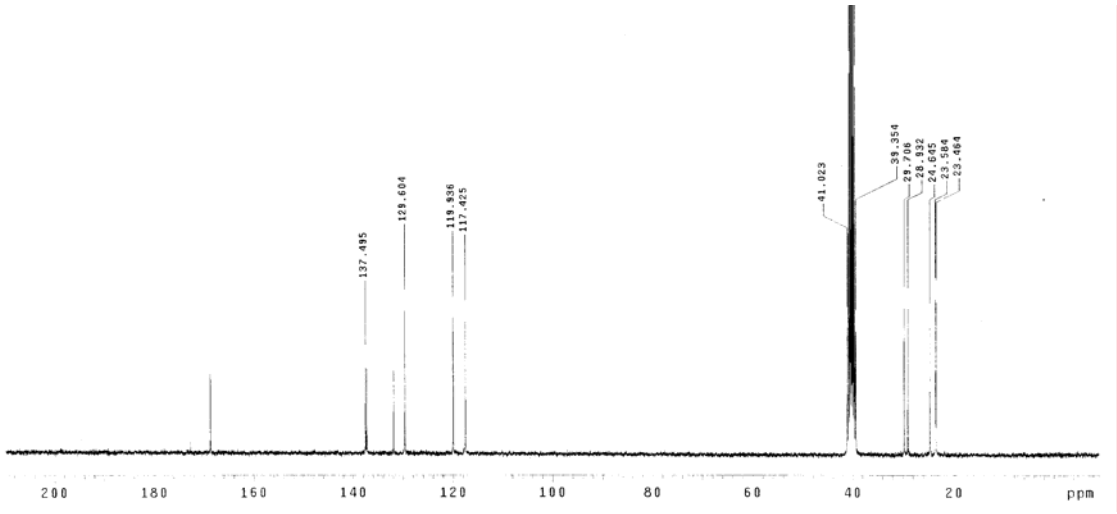

156.377
138.871
137.522
134.024
77.739
77.311
76.891
200
180
160
140
120
100
80
60
40
20
ppm

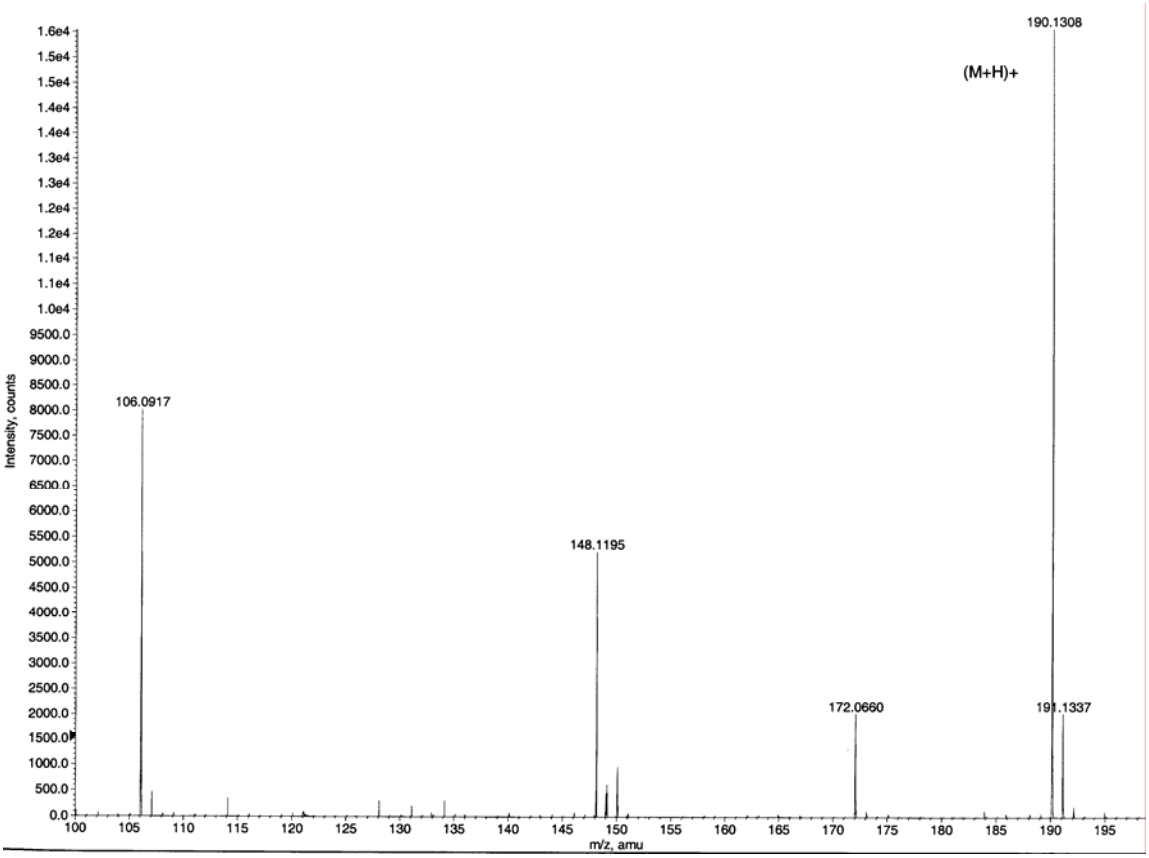

190.1231
Intensity, counts
m/z, amu

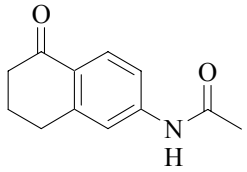

O
N
H

42

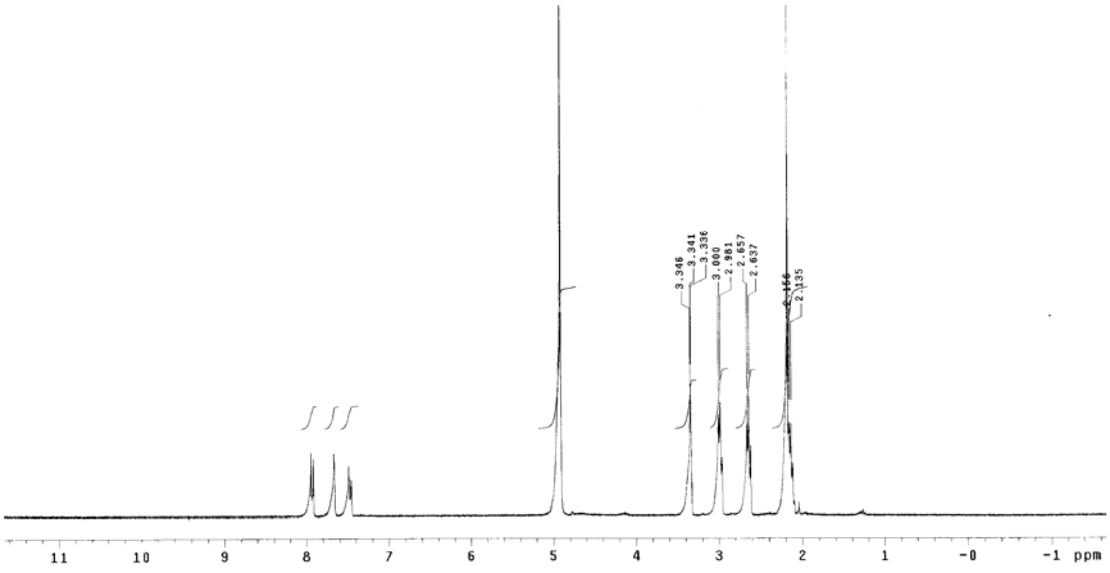

2.723
2.110
2.103
2.096
1.787
1.776
11
10
9
8
7
6
5
4
3
2
1
-0
-1 ppm

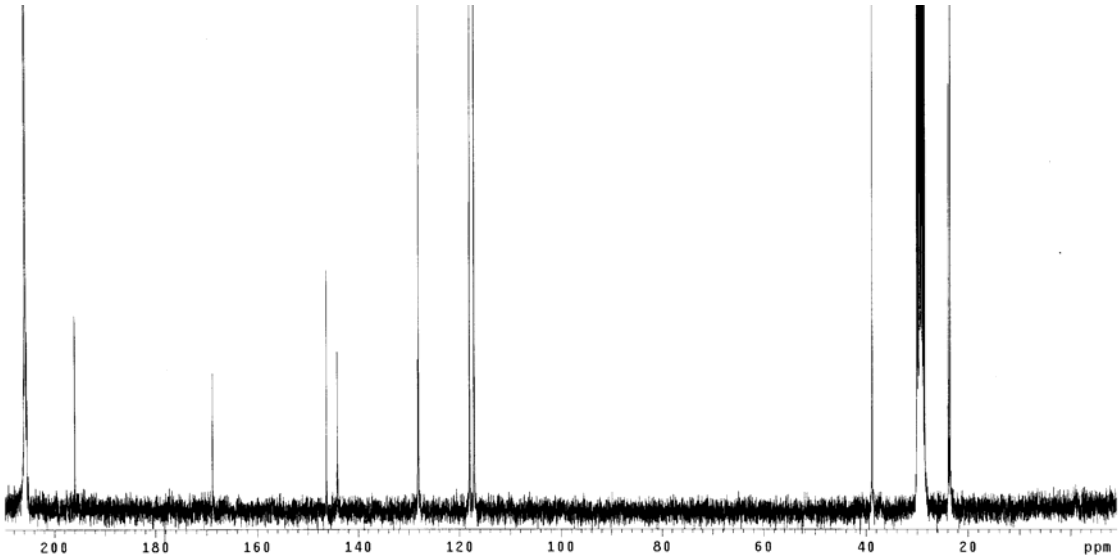

137.495
129.604
119.836
117.425
41.023
39.354
29.706
28.832
24.645
23.584
23.464
200
180
160
140
120
100
80
60
40
20
ppm

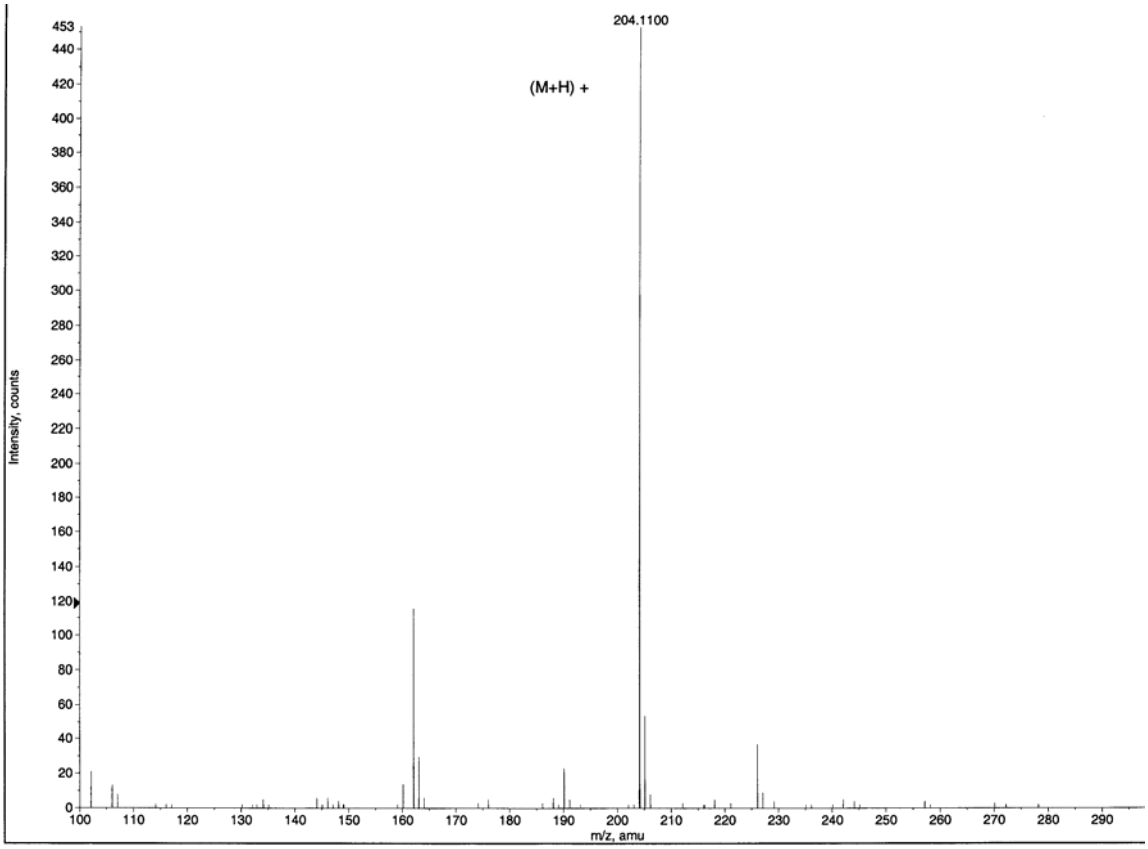

190.1308
(M+H)+
106.0917
148.1195
172.0660
191.1337
Intensity, counts
m/z, amu

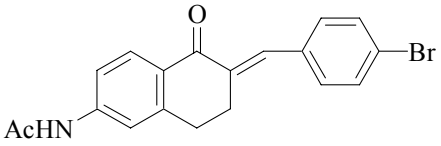


43

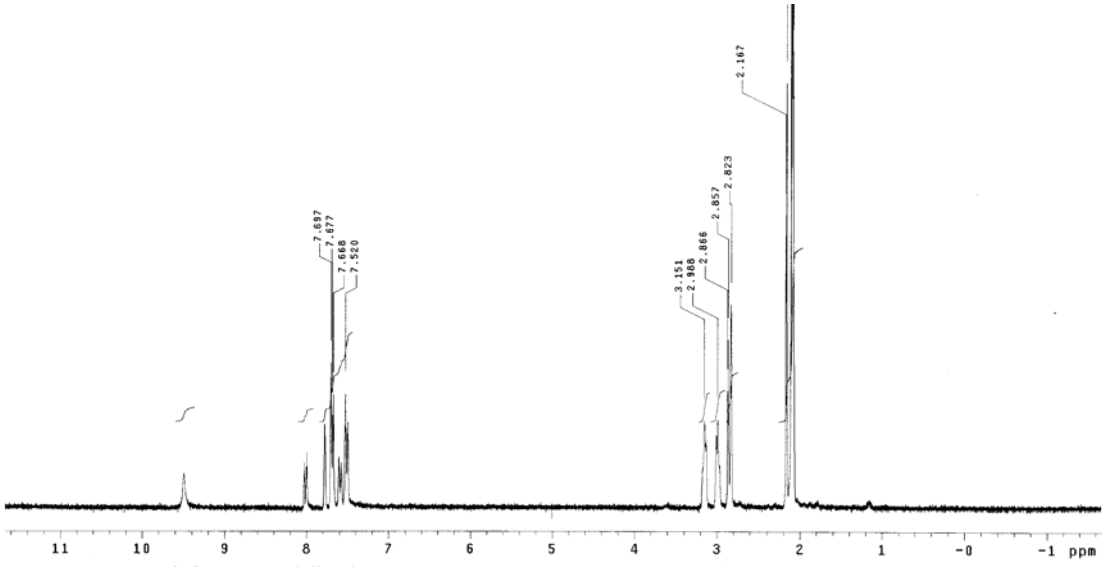

3.346
3.341
3.336
3.000
2.981
2.657
2.637
2.135
11
10
9
8
7
6
5
4
3
2
1
-0
-1 ppm

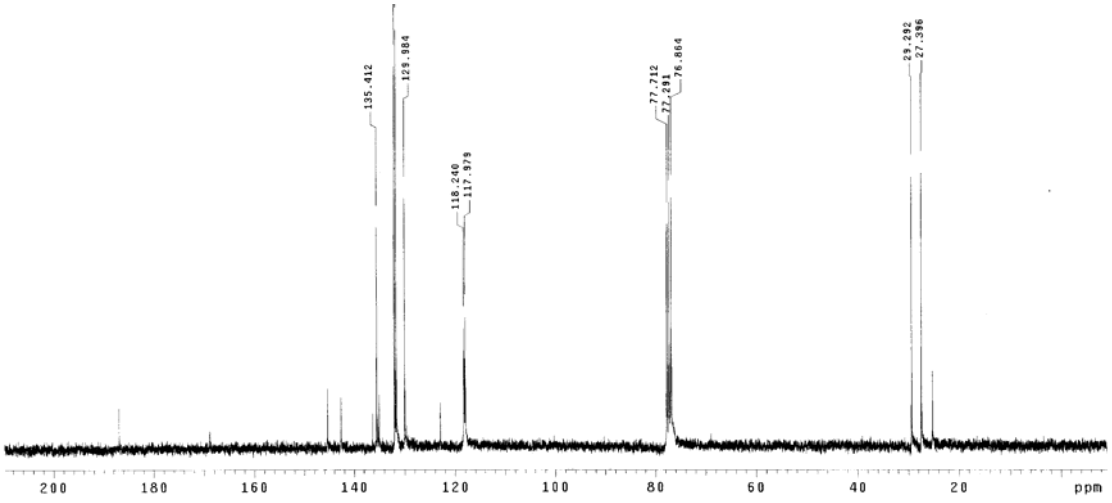

200
180
160
140
120
100
80
60
40
20
ppm

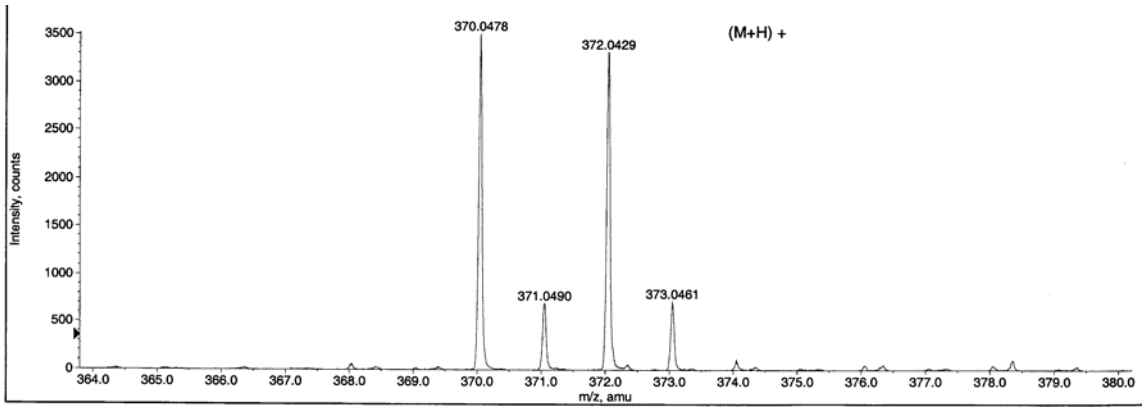

204.1100
(M+H) +
Intensity, counts
m/z, amu

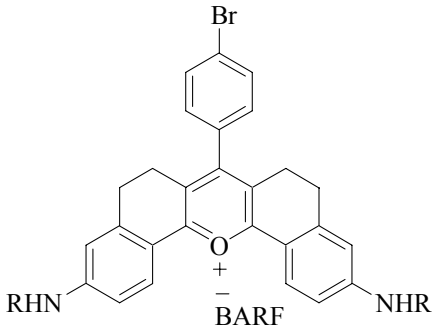

O
Br
AcHN

45


7.697
7.677
7.668
7.520
3.151
2.988
2.866
2.857
2.823
2.167
11
10
9
8
7
6
5
4
3
2
1
-0
-1 ppm

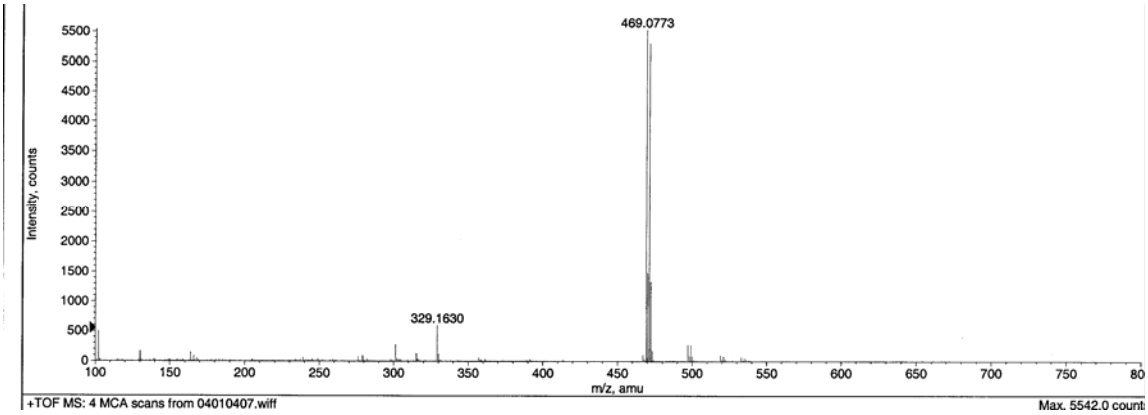

135.412
129.884
118.240
117.979
77.712
77.291
76.864
29.292
27.396
200
180
160
140
120
100
80
60
40
20
ppm

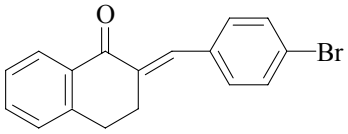

370.0478
372.0429
(M+H) +
371.0490
373.0461
Intensity, counts
3500
3000
2500
2000
1500
1000
500
0
364.0
365.0
366.0
367.0
368.0
369.0
370.0
371.0
372.0
373.0
374.0
375.0
376.0
377.0
378.0
379.0
380.0
m/z, amu


Br
O
+
RHN
BARF
NHR

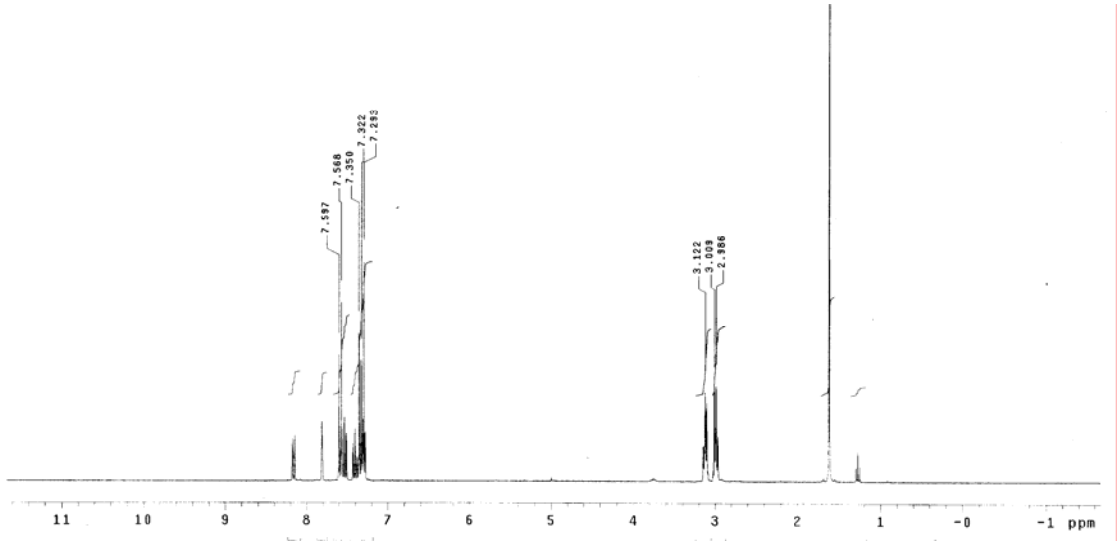
R = H, Ac

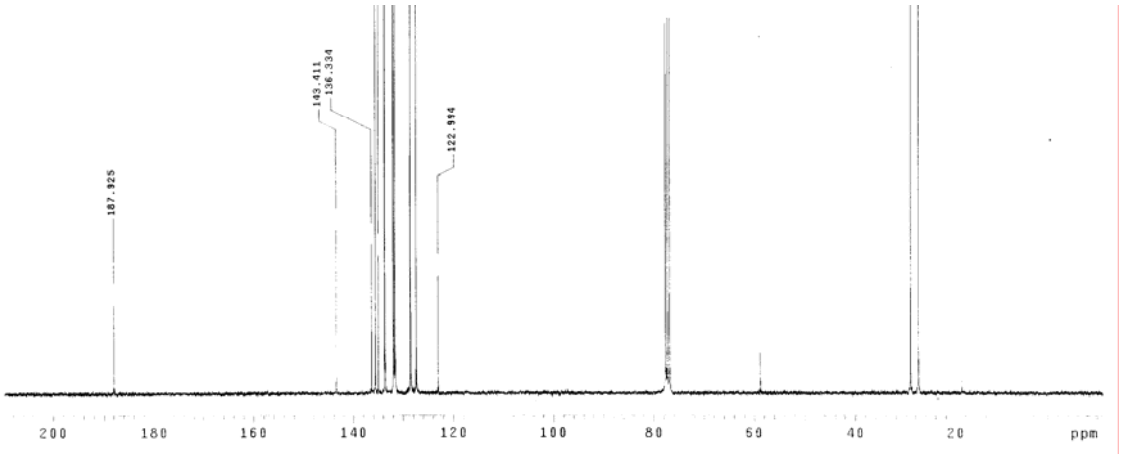
46

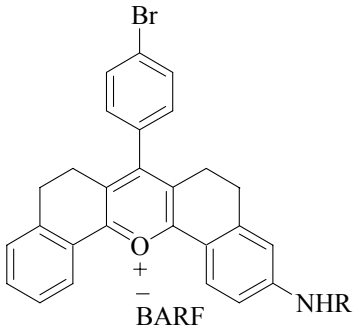

469.0773
329.1630
Intensity, counts
m/z, amu
+TOF MS: 4 MCA scans from 04010407.wiff
Max. 5542.0 counts


O
Br

48

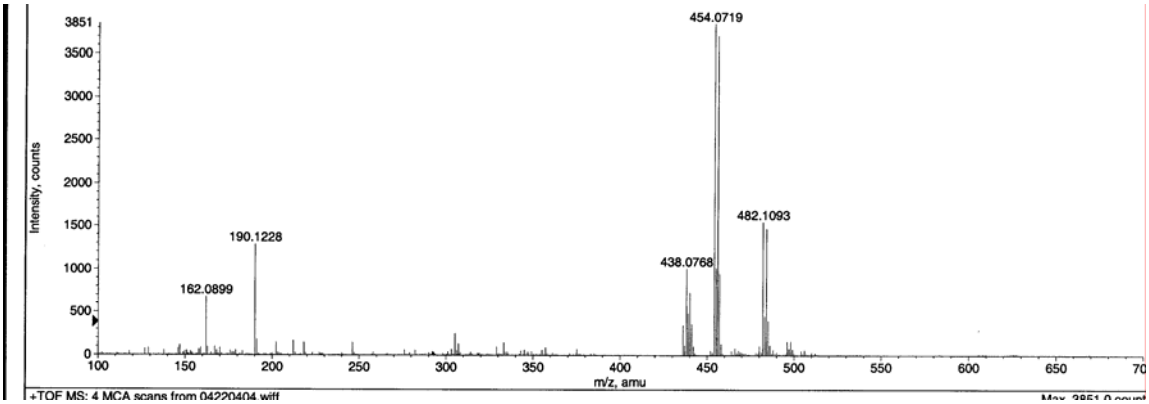

7.587
7.568
7.550
7.322
7.293
3.122
3.009
2.986
11
10
9
8
7
6
5
4
3
2
1
-0
-1 ppm


197.325
143.411
136.334
122.914
200
180
160
140
120
100
80
60
40
20
ppm


Br
O
+
–
BARF
NHR

R = H, Ac

49


454.0719
482.1093
190.1228
438.0768
162.0899
Intensity, counts
m/z, amu
+TOF MS: 4 MCA scans from 04220404.wiff

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